University of Alberta

A role for PI3K in the Cytoskeletal Rearrangements Required for CTL Degranulation

By

Laura R. Mireau

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In Immunology

Department of Medical Microbiology and Immunology

Edmonton, Alberta

Fall, 2002

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Sept 25, 02

Laura R. Mireau

#605-1 Michener Park NW
Edmonton, Alberta
T6H 4N1

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"A role for PI3K in the Cytoskeletal Rearrangements required for CTL Degranulation"** submitted by Laura R. Mireau in partial fulfillment of the requirements for the degree of Master of Science in Immunology.

Dr. Hanne L. Ostergaard
(Supervisor)

Dr. Larry Guilbert

Dr. Deborah N. Burshtyn

Dr. Andrew R. E. Shaw

Sept 20, 2002

Abstract

Cytotoxic T lymphocytes (CTL) are immune effector cells that function to detect and destroy virally infected and cancerous cells. CTL become activated to kill as a result of signals initiated by T cell receptor (TCR) engagement. TCR signalling induces CTL degranulation in part by initiating cytoskeletal changes, which contribute to T cell/target cell adhesion, sustained signalling, receptor localization and stabilization, and polarization of cytolytic granules. Although these cytoskeleton-dependent events are required for CTL effector function, the pathways that link signalling events to the cytoskeleton are unclear.

The lipid kinase phosphatidylinositol-3 kinase (PI3K) has been implicated in cytoskeletal rearrangements. This study shows that PI3K activity is required for signalling events that mediate CTL degranulation. Moreover, we show a role for PI3K in the phosphorylation of paxillin, adhesion, cell spreading, and in the phosphorylation of PKC θ. Together, the results of this study suggest that PI3K contributes to CTL degranulation by linking membrane proximal signalling to cytoskeletal rearrangement.

Acknowledgements

I'd first and foremost like to acknowledge my supervisor Hanne Ostergaard for believing in me and for giving me the chance to prove myself. Hanne, your guidance and encouragement has inspired in me, a love of immunology and helped me to discover an unexpected passion for research.

To my lab mates, Troy, Lawrence, Bindy, Leslie, Stacey, Barbara, Tara, and especially Kara, thanks for making the lab environment amazing. It has been a gift to work with such wonderful, positive and caring people, people I will always think of as friends.

I'd also like to thank Larry Guilbert for all his words of wisdom and continuous encouragement throughout the last few years. As well I'd like to acknowledge his time spent in mentorship with regard to teaching and the UTS program.

To Debbie Burshtyn, thanks for patience in mentoring a "wanna-be" teacher, and for providing insight and a kind ear to an uncertain graduate student who was trying to find her way.

To the Kane lab, thanks for being great neighbors and caring friends.

To my friend Lori, thanks for being a good friend, for remaining positive through personal trial and for caring about my progress in life and as a masters' student.

To Robyn, thanks for being proud of me, and for treating me like family.

For Peter, who pushed and pushed till I almost broke, but at the same time cared enough to help me keep it together, and inspired me to walk away stronger for it in the end.

For Pete

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List of Abbreviations

APC	Antigen presenting cell
Arp	Actin-related protein
ATCC	American Type Culture Collection
BH	Break point cluster region-homology domain
BLT	Benzyloxy carbonyl-L-lysine Thiobenzyl Ester
Btk	Brutons Tyrosine Kinase
CD	Cluster Designation
CMI	Cell Mediated Immunity
CTL	Cytotoxic T lymphocyte
Cyto-D	Cytochalasin D
DAG	Diacyl Glycerol
DNTB	Dithio-bis2-nitrobenzoic acid
ECC	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ERK	Extracellular Regulated Kinase

FAK	Focal Adhesion Kinase
FCS	Fetal Calf Serum
FITC	Fluorescence isothyocyanate
Fyn	Src kinase p59
GSK	Glycogen Synthase Kinase
HRP	Horse Radish Peroxidase
ICAM-1	Intercellular Adhesion Molecule
IL	Interleukin
IP	Immunoprecipitate
IP3	Inositol Tris-phosphate
ITAM	Immuno-tyrosine based Activation Motif
KO	Knock out
LAT	Linker for Activation of T cells
Lck	Lymphocyte-specific Cytoplasmic protein Tyrosine Kinase
LFA-1	Leukocyte Function Associated Antigen

MAP	Mitogen Activated Protein Kinase
MHC	Major Histocompatability Complex
MTOC	Microtubule Organizing Center
NFAT	Nuclear Factor for Activated T cells
OD	Optical Density
PAK	p21 Activated Kinase
PBS	Phosphate Buffered Saline
PDK-1	PI3K-dependent Kinase-1
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-Kinase
PIP	Phosphatidylinositol-phosphate
PKB (AKT)	Protein Kinase B
PKC	Protein Kinase C
PLC γ	Phospholipase C gamma
PMA	Phorbol-12 Myristate Acetate
PMSF	Phenylmethylsulfonyl fluoride
RSB	Reducing Sample Buffer

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH	Src homology
SMAC	Supramolecular Activation Cluster
SLP-76	SH2 domain containing leukocyte protein
TCR	T cell receptor
WASp	Wiscott Aldridge Syndrome protein
ZAP-70	ζ associated protein

Chapter I

Overview of CTL Effector Function and Activation

1.1 General Role in Immune response

Human survival requires that the body have the ability to protect itself against deleterious alterations that result from pathogenic infection or mutation. This protection is conferred in part by the immune system. As a consequence the immune system must be able to distinguish normal self from what is altered or foreign.

1.1.1 The Immune System

The immune system is compartmentalized into two branches: the innate immune response and the adaptive immune response. The two branches work in a highly coordinated fashion, covertly communicating with each other strategies for the control and elimination of pathogens. The innate branch consists of populations of leukocytes that respond *en masse* upon initial pathogenic invasion. Leukocytes contribute both directly and indirectly to pathogen clearance. Directly, infiltrating leukocytes mediate initial destruction of infected tissues via cytolysis. Indirectly, leukocytes that have encountered antigen contribute to pathogen clearance by secreting chemical mediators that provide information to the adaptive immune response on the type of adaptive defense required. Later in infection, the adaptive response translates the signals from the innate response and uses the information to design and implement a highly specific response. Lymphocytes, the cells that

comprise the adaptive immune system, respond only to specific antigens; lymphocytes are specifically selected by interaction with their antigen to become activated and to expand. Only those lymphocytes that see their respective antigen are activated and expanded, thus only those cells that have been altered to express the foreign antigen (for which the selected lymphocytes are specific) will be targeted and destroyed. In this way the adaptive immune system generates a specific immune response for a specific pathogen. As a means of achieving this specificity, the adaptive immune system is sub-divided into two branches; the humoral branch, which is activated and functions to safeguard primarily against extracellular pathogens, and the cell mediated immunity (CMI) branch, which functions in defense primarily against intracellular pathogens. The principal cellular players in the humoral and CMI branches are B and T cells respectively. T cells are considered the centre of the adaptive response in that they can functionally regulate the activity of all other cell types. T cells can be generally subdivided into $CD4^+$ T helpers and $CD8^+$ cytotoxic T lymphocytes (CTL). Where T helpers play a key role in immune regulation, CTL play a key role in the direct destruction of infected and cancerous target cells.

1.1.2 CTL Characterization and Activation

Cytotoxic T lymphocytes (CTL) are critical effector cells in the adaptive immune response that function to directly destroy cells infected with intracellular pathogens, or cells that have been transformed. Generally CTL are characterized as lymphocytes that express a T cell antigen receptor

(TCR) in concert with CD3, CD8, and Thy-1 on their surface. Activation of CTL results when a naïve $CD8^+$ T cell is stimulated through interaction of the T cell receptor (TCR) with foreign antigen in the context of major histocompatibility complex (MHC) class I expressed on a target cell. TCR engagement triggers the expression of cytokine receptors (i.e. interleukin 2 and 6 (IL2 and IL6)) that recognize cytokines derived from T helper cells. Helper cytokines stimulate the $CD8^+$ T cells to proliferate and differentiate into effector CTL and memory cells. Only those cells that have "seen" their antigen presented in the context of MHC class I become activated. In this way the adaptive immune response selects and expands immune cells that are specific for, and effective at recognizing, specific antigens.

1.1.3 CTL Function

The predominant function of CTL is to directly destroy altered-self or infected cells (1). CTL are uniquely designed to control intracellular pathogens because in general, they are triggered by intracellular protein-derived peptides, loaded onto class I MHC molecules which are expressed on all cells in the body. Their granularity as well as the expression of FAS ligand on their surface mediate their killing function; CTL seek out non-self, or altered self-cells and destroy them via degranulation, or via FAS ligand mediated killing (2). Both degranulation and FAS ligand expression trigger target apoptosis. In the case of a virally infected target, apoptosis interferes with intracellular viral propagation resulting in attenuation of viral infection. Cancerous cells, if recognized as altered-self cells by CTL through the

expression of altered endogenous peptides in the context of MHC class I, are eliminated before malignancy and metastasis can occur.

Kees *et al.* first demonstrated the importance of CTL in immune defense against intracellular pathogens in 1976 (3). They showed that transfer of specific cytotoxic T cells to mice infected with ectromelia virus results in a reduction in virus titre within target organs (liver and spleen) of the virus. Later Yap *et al.* (4) showed, through adoptive transfer experiments, that CTL protect host mice from death and dramatically reduce virus titres when transferred to influenza-infected mice. With regard to CTL defense against malignancy, in 1977, Coates and Crawford showed not only that human melanoma cells could be transplanted to nude mice, but also that CTL from the tumour donor could inhibit the growth of the tumour graft in the recipient mice (5). This evidence initiated what has since been firmly established; a role for CTL in intracellular pathogen clearance and cancer control.

CTL can also contribute to autoimmune disorders if their activity becomes non-specific or if CTL are generated that express TCR specific for self-peptides. Under these circumstances, CTL are potent destroyers of healthy tissue. The key role of CTL in autoimmunity has been demonstrated in mice; mice with a deficiency in the CTL degranulation pathway have dramatically less severe forms of induced diabetes (6) (7).

1.2 T cell Activation

CTL are highly potent killers, thus "on switches" and checkpoints are required to prevent aberrant T cell activation. In order for a T cell to become activated *in vivo*, antigen specific receptors as well as non-antigen specific receptors on the T cell membrane must engage ligands on a target cell or antigen-presenting cell (APC) in a highly coordinated manner. The requirement for coordination of receptor engagement is a key example of the "built-in" regulatory mechanisms involved in CTL activation. The critical surface molecules on the T cell, important for T cell activation, are the T cell receptor (TCR), the co-stimulatory molecule CD28, integrins such as leukocyte function associated antigen-1 (LFA-1), and CD8. Coordinated engagement of these molecules with their ligands, results in T cell activation.

1.2.1 T cell Receptor (TCR)

The central receptor for stimulation of a T cell is the TCR. The TCR is described as a somatically rearranged, clonal antigen receptor (8). Every individual T cell somatically rearranges a unique TCR thus, the overall repertoire of T cells have vast TCR diversity (9) while individual T cells are highly specific for a limited range of peptide antigens. Any given TCR on a particular CTL is specific for an intracellular antigen-derived peptide presented in the context of MHC class I.

The TCR is a multi-subunit transmembrane glycoprotein composed of α and β chains, the CD3 complex and TCR ζ. The α and β chains directly bind antigenic peptide and self-MHC, and thus dictate specificity of TCR

recognition (10) (11). The invariant CD3 complex, which is composed of non-polymorphic, transmembrane, heterodimeric proteins CD3γ/ε, CD3δ/ε, and usually one homodimer of TCR ζ/ζ (12) (11), is required to transduce signals in response to TCR (α/β) engagement with MHC/peptide; the α and β chains have no intrinsic signal transduction capabilities.

Although the TCR interacts with MHC/peptide with great specificity, the affinity of the interaction is very low. Even so, TCR/MHC/peptide interaction is absolutely required for T cell activation and effector function. Signaling through the TCR initiates the majority of T cell responses required for degranulation. These include most membrane proximal signaling events that facilitate transcription of proteins such as IL2, as well as cytoskeletal rearrangements such as cell spreading and microtubule organizing center (MTOC) polarization.

1.2.2 CD28

Signals generated by the TCR are amplified by the concurrent signaling of costimulatory molecules. The primary costimulatory interaction involved in naïve T cell activation is the engagement of CD28 with its ligand B7-1 or B7-2 (CD86 and CD80) on an APC. Costimulation by ligand interaction between CD28 and B7 is required for T cell proliferation and differentiation as shown through antibody blocking and cross-linking experiments (13). CD28 contributes to T cell activation by functioning both as an adhesion molecule, and as a signaling molecule (14). As an adhesion molecule, CD28 binding B7 helps stabilize the interaction between the T cell

and the target cell, allowing for low affinity TCR/MHC/peptide interactions to occur. CD28 signaling provides a crucial second signal for TCR regulated cytokine production and proliferation (15) (16) (17). Because CD28 shares many of its biochemical functions with the TCR, CD28 signaling is thought to intensify and prolong signals normally generated by the TCR.

CD28 may also regulate aspects of cytoskeletal rearrangement that occur upon T cell activation. Wulfing and Davis suggest that CD28 costimulation signals the action of myosin motor proteins to reorient molecules attached to the actin cytoskeleton towards the target cell interface. Thus, engagement of CD28 contributes to cytoskeletal rearrangement by promoting the cytoskeletal–dependent recruitment of cell surface proteins (18) and lipid rafts rich in kinases and adaptor proteins (19).

1.2.3 LFA-1

Because the interaction between the TCR/MHC/peptide is very low affinity, adhesion molecules are required to initiate and maintain cell/cell contact. This initial contact or adhesion plays a key role in T cell proliferation (20) (21) and T cell mediated killing (22) by facilitating TCR surveillance of the target cell for antigen. Adhesion is mediated by the engagement of adhesion molecules LFA-1 on the T cell surface with its ligand intercellular adhesion molecule (ICAM) on the target cell. The importance of the LFA-1/ICAM interaction has been illustrated by experiments showing that T cell activation is inhibited when LFA-1/ICAM interactions are blocked using antibodies to LFA-1 (23) (24) (25).

LFA-1 is member of the β2 integrin family and as such, is a glycoprotein heterodimer composed of transmembrane α and β subunits noncovalently associated by their extracellular domains with very short cytoplasmic tails (22) (26). LFA-1 is expressed on all leukocytes (22) and functions as an adhesion molecule upon interaction with ICAM-1 (27), ICAM-2 (28) and ICAM-3 (29).

Both low and high avidity forms of LFA-1 exist (30); the avidity of integrins for their ligands depends on the activation state of the cell (31). In T cells, signaling through the TCR or CD28 induces integrin activation and increased adhesion through a process called inside-out signaling (32) (33) (34). Increased adhesion of LFA-1 for ICAM mediates prolonged cell/cell contact, thus stabilizing TCR and CD28 engagement with their respective ligands, resulting in T cell activation.

LFA-1 also contributes to the cytoskeletal rearrangement required for T cell activation in that it co-localizes with and triggers the re-localization of talin at sites of antigen specific cell-cell contact (35) (56), is associated with α-actinin (36), and with vinculin (36) (37).

1.2.4 CD8

Another surface molecule, CD8, plays an important role in CTL activation and proliferation by participating in coreceptor function (12). CD8 interacts with the same MHC as the TCR and therefore is thought to stabilize the TCR/MHC interaction. Experiments where CD8 is sequestered away from the TCR upon T cell/APC interaction result in diminished T cell

responses (38) (39). As well, blocking CD8/MHC interaction inhibits CTL degranulation (40). Interestingly, CD8 can also undergo triggered binding and contribute to adhesion by engaging non-antigenic class I MHC (41) (40) however the mechanism of this enhancement is largely unknown.

1.2.5 Summary of T cell Activation

T cell activation occurs in response to the coordinated engagement of receptors on the T cell surface. The receptor molecules involved in T cell activation include the antigen specific TCR, the co-stimulatory molecule CD28, the adhesion molecule LFA-1, and the coreceptor molecule CD8. Receptor/ligand engagement results in T cell activation and effector function.

1.3 Cytoskeletal Rearrangement

Receptor ligand interactions result in T cell activation and effector function in part by initiating the dynamic reorganization of the cytoskeleton. In general, the cytoskeleton gives the cell shape, asymmetry, specific membrane organization, polarity for movement, and provides a scaffold for signaling components upon T cell activation. Experiments in T cells where the actin cytoskeleton has been disrupted using cytochalasin D (cyto-D) illustrate the importance of an intact and dynamic cytoskeleton for T cell activation; T cell activation is abrogated with cyto-D treatment (42). Other research has shown that sustained calcium flux, required for activation, depends on the reorganization of the actin cytoskeleton (43).

The specific contributions of the cytoskeleton to T cell activation are many fold. The actin cytoskeleton 1) provides scaffolds and regulation for integrin-mediated adhesion (44) (45), 2) provides signaling scaffolds and increases the concentration of intracellular molecules in proximity to the TCR (42) (46) (47), 3) recruits or stabilizes glycolipid rich rafts that are concentrated in the vicinity of engaged TCR (19), and 4) plays a role in receptor clustering and immune synapse formation, thus increasing the likelihood of TCR/MHC interactions (48).

Tubulin may also play a role in activation since polarization of the MTOC is required for effector function as it establishes polarity of the golgi-complex and associated secretory organelles, thus directing secretion of cytokines and granules (49) ((50) (51).

1.3.1 The Cytoskeleton and Adhesion

T cell activation results from relatively low affinity interactions between the TCR and MHC/peptide. In order for TCR/MHC/peptide interactions to occur, adhesion molecules must tether the T cell to an APC in a transient fashion allowing the TCR to survey the target for the appropriate antigen. If the right antigen is present, TCR and coreceptor signaling results in T cell activation. Thus, adhesion of the T cell to an APC is critical for initiating T cell activation and effector function.

In resting T cells, LFA-1, the principal T cell adhesion molecule is tethered to the actin cytoskeleton and maintained in a low activity state (44)

(45) (52)). Undefined, non-antigen specific activation signals induce T cell polarization and the release of the inhibitory cytoskeletal interaction so that LFA-1 can diffuse freely in the plane of the membrane (44). New actin polymerization is concentrated in leading protrusions of the cell (53); highly mobile LFA-1, as it binds ICAM on an APC, forms a new adhesion stabilizing interaction with actin (54) in a process thought to involve α-actinin (55), talin and vinculin (56) (57) (58). Talin is thought to help stabilize clusters of LFA-1 (56) (57) as it couples integrins to F actin.

It is thought that adhesive mechanisms such as LFA-1/ICAM-1 engagement are required to overcome the barrier of close cell-cell contact imposed by the negatively charged glycocalyx of the T cell and APC (14). Thus multiple low affinity, adhesion interactions allow cells to be brought close enough together for the TCR to engage the MHC/peptide (14), which interact when the cells are only ~ 15 nm away from each other, as opposed to the 50-100 nm separation imposed by molecules such as mucin CD43. (59) (10) (60)

1.3.2 Inside-out Signaling

Signaling through the CD3 complex results in increased affinity of LFA-1 for ICAM-1, which tightens and stabilizes the interaction between the T lymphocyte and the APC, thus facilitating activation. This response has been termed inside-out signaling as it was first described for LFA-1/ICAM-1 interactions (32). Increased avidity between LFA-1 and ICAM-1 is required for T cell activation and for effector function. Recent work in defining the

signalling pathways that comprise inside out signalling has identified a cytoplasmic regulatory factor in integrin activation called cytohesin-1. Cytohesin-1 is a 47-KDa, intracellular protein that interacts with the cytoplasmic domain of LFA-1 and has been shown to enhance LFA-1 binding to ICAM-1 upon association (26). Although cytoshesin-1 likely plays a key role in inside-out signalling, the upstream events that facilitate cytohesin-1/LFA-1 interaction upon TCR signalling are poorly understood. Moreover initial adhesion and inside-out signaling are absolutely required for T cell activation and effector function; however the signaling processes that trigger these cytoskeleton dependent processed have yet to be fully elucidated.

1.3.3 The Cytoskeleton and Sustained Signaling

In order for cytoplasmic proteins to participate in signaling cascades, they must be recruited to the membrane and held in place to gain access to upstream kinases as well as downstream targets. The cytoskeleton plays a major role in this recruitment and stabilization. Many important signaling receptors (TCR ζ chains) as well as cytoplasmic proteins bind the cytoskeleton upon TCR signaling, resulting in protein clustering and phosphorylation. Understandably, the cytoskeleton must be intact for the sustained phosphorylation required for T cell activation to occur (61) (42); tyrosine phosphorylation is transient in cells in which the cytoskeleton has been disrupted using cytochalasin D (Chen and Ostergaard, H., manuscript in preparation). As well, sustained tyrosine phosphorylation is correlated with

cytoskeletal rearrangement; T cells stimulated with immobilized anti-CD3 undergo dramatic morphological changes concurrently with sustained tyrosine phosphorylation of membrane proximal proteins, whereas T cells, stimulated with cross-linked anti-CD3 do not spread, nor undergo sustained phosphorylation of membrane proximal proteins (61). Thus it is clear that sustained signaling is dependent on an intact and dynamic cytoskeleton, however, the nature of this dependence has yet to be clarified.

1.3.4 The Cytoskeleton and Lipid rafts

Recent research has provided evidence for the existence of specialized membrane micro-domains enriched in cholesterol and sphingolipids (62) (63). These molecules spontaneously form liquid-ordered membrane regions, which are insoluble in non-ionic detergents, and have been coined "lipid rafts" (63) (64). Important cytoplasmic signalling molecules are targeted to rafts by acylation with saturated fatty acyl moieties (palmitoyl, myristoyl), which link proteins to the cytoplasmic lipid leaflet or microdomain (65).

Extensive cross-linking of the TCR with antibodies promotes rapid activation of Src kinases and subsequent accumulation of newly tyrosine-phosphorylated substrates in rafts (66) (67) (68), including CD3ζ molecules (69) (70) (71) ζ associated protein (ZAP-70), phospholipase C gamma (PLCγ1), phosphatidylinositol 3-kinase (PI3K), Vav, (69) and linker for activated T cells (LAT) (72) (73) (74) (66). Thus, lipid rafts are proposed to play an essential role in T cell signal transduction by functioning as signalling

scaffolds and contributing to sustained signalling (70) (19) (74) (75) (76). The importance of rafts in T cell activation is evidenced by experiments showing that disruption of lipid rafts attenuates T cell activation (77). Upon T cell activation, the actin scaffold recruits and stabilizes lipid rafts thus facilitating the key role of lipid rafts in cell signalling and T cell activation (70) (19) (78).

1.3.5 Immune synapse formation

When agonist MHC–peptide complexes on an APC contact the TCR, the T cell actin cytoskeleton coordinates distinct relocalization of adhesion molecules, antigen receptors, and cytosolic proteins. Actin filaments play a key role in regulating molecular movements on the cell surface (18) (79) resulting in the formation of the immunological synapse; molecules organized into distinct supramolecular activation clusters (SMACs) (79) (58). During the process of SMAC formation, molecules at the contact site between the T cell and the APC resemble that of a bulls-eye. After approximately 5 minutes of engagement between a CTL and target cell, TCR/MHC/peptide complexes together with cytosolic Protein Kinase C θ (PKC θ) have migrated to the centre of the SMAC (c-SMAC) while adhesion molecules (LFA-1 and ICAM) have migrated to the periphery (p-SMAC) (80). The contribution of the immune synapse to T cell activation is unclear, however, synapse formation may facilitate 1) localization of receptors to increase adhesion, 2) presentation of a polarized surface for biochemistry, and 3) clear segregation of specific molecules to particular zones (i.e. CD45), among other possibilities. In CTL, the immune synapse likely functions to direct CTL

degranulation (80). Apart from function, it is clear that the immune synapse forms upon T cell activation, however, the signalling events that trigger cytoskeletal rearrangement and the mechanism of cytoskeletal rearrangement remain to be elucidated.

1.3.6 CTL Degranulation and MTOC polarization

One of the dominant mechanisms of contact-dependent lymphocyte–mediated cytotoxicity is granule exocytosis or degranulation (81). Degranulation requires polarization of the MTOC as it establishes polarity of the Golgi complex and associated secretory organelles, thus directing secretion of cytokines and granules (82) (49) (50) (51). Upon encounter with a target, a killer cell reorients its granules to the region of receptor activation, along with the MTOC, and releases the granule components into the region of contact between the two cells. The directed reorientation of granules grants specificity to the perforin mediated pathway (50). Although MTOC polarization is known to be downstream of TCR signaling, and required for effector function (83) (84), the signaling cascade that mediates polarization is not fully understood.

1.3.7 Summary of Cytoskeletal involvement in CTL Activation

The cytoskeleton is required for many process involved in T cell activation, including adhesion of a CTL to a target cell, recruitment of signaling molecules and facilitation of sustained signals through direct interaction with actin, or via organization of lipid rafts. As well the

cytoskeleton coordinates the formation of the immunological synapse, and orchestrates MTOC polarization thus facilitating degranulation.

1.4 T cell Signaling

The engagement of receptors on the surface of a T cell results in the initiation of a number of biochemical pathways. These pathways or cascades cooperate leading to various cellular responses including cytoskeletal rearrangement.

1.4.1 TCR Signaling

TCR engagement with MHC/peptide triggers a myriad of signalling cascades (Figures 1 and 2). Signalling starts with the CD3; each chain of the CD3 complex including the TCR ζ chains, contains at least one immunoreceptor-tyrosine-based activation motif (ITAM) in the cytoplasmic region. ITAMs consist of the amino acid sequence $YXXLX_{(6-8)}YXXL$ and are the key components for membrane proximal signal transduction (12) (11). Src-family kinases such as lymphocyte-specific cytoplasmic protein-tyrosine kinase (Lck) or src kinase p59 (Fyn) initiate signalling by phosphorylating the ITAMs on the TCR ζ chain (85) (86). Phosphorylation of ζ leads to recruitment of multiple signalling proteins, including ZAP-70 (85) (86), via tandem src-homology 2 (SH2) domains.

ZAP-70 binding the phosphorylated ζ chain initiates its phosphorylation and activation (87). ZAP-70 activation is essential for downstream signalling events that result in cytokine production (88) as well as MTOC polarization

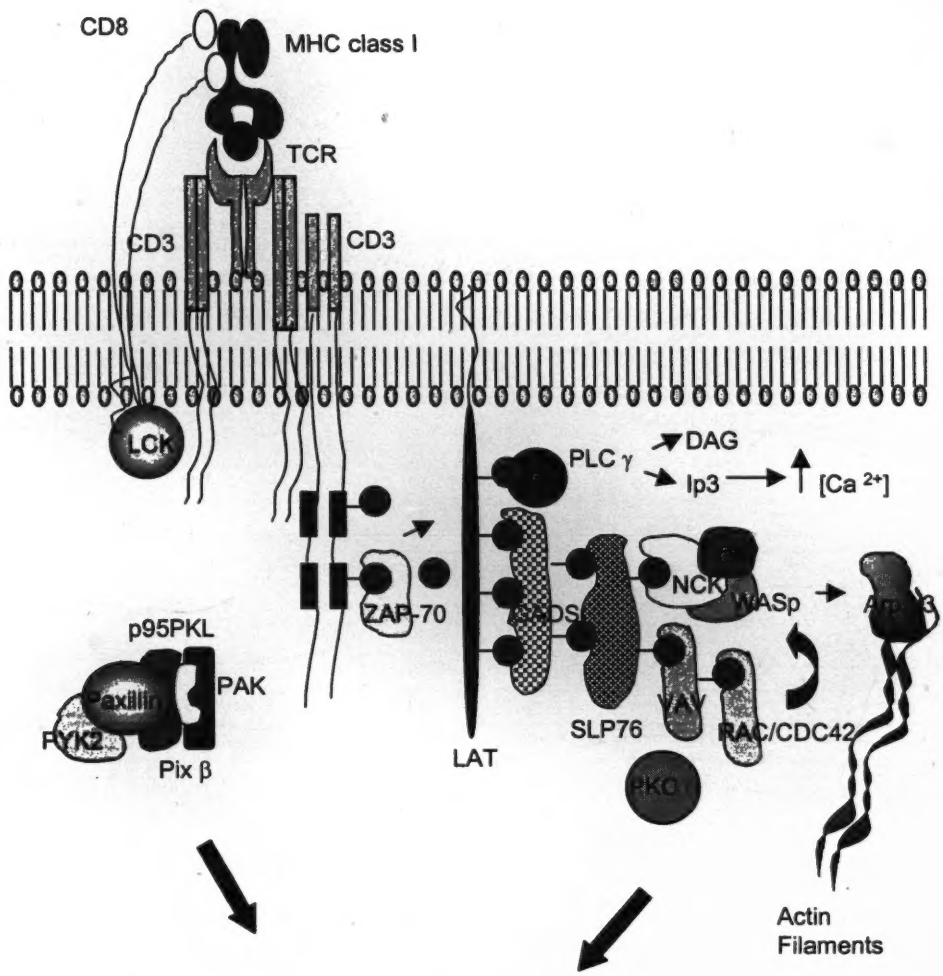


Figure 1. **Signaling cytoskeletal rearrangements through the TCR.** TCR engagement triggers the activation of LCK which phosphorylates the ζ chains of the TCR. Phosphorylation of the ζ chains facilitates the recruitment and activation of ZAP 70, which correspondingly phosphorylates LAT. LAT acts as a docking site for many of the molecules involved in actin polymerization and cytoskeletal rearrangement.

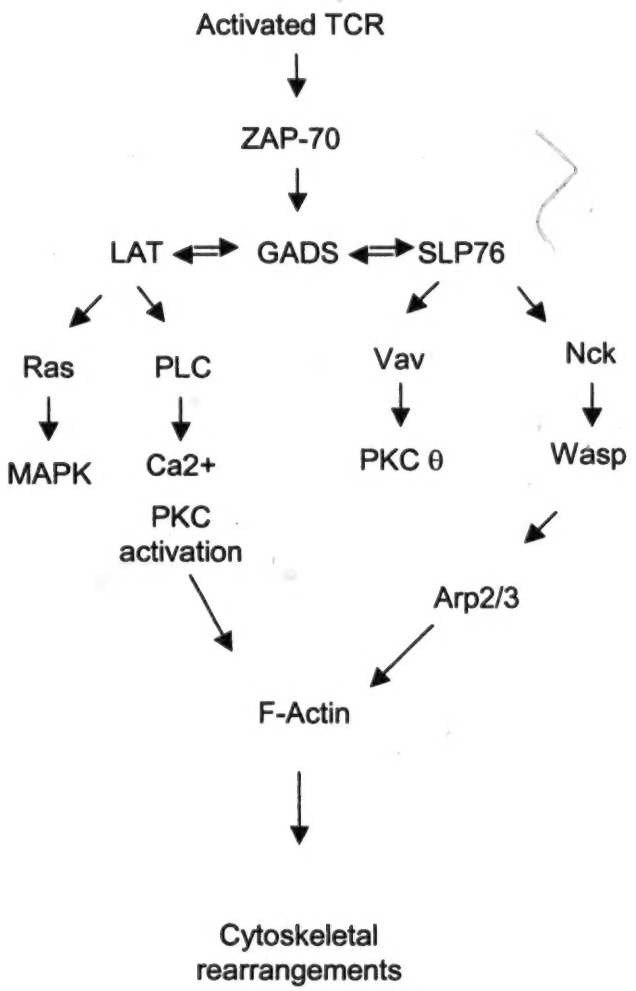


Figure 2. **TCR stimulated actin polymerization.**

(84). One of ZAP-70s activities is to phosphorylate LAT and SH2 domain containing leukocyte protein (SLP-76) (89) (90) (91) (92), which in turn, serve as docking sites for downstream signalling proteins. Cytoplasmic proteins that bind LAT and SLP-76 gain access to upstream kinases and downstream proteins.

SLP-76 provides a docking site for Tec kinase, which phosphorylates the LAT-associated protein PLC γ. PLC γ signals the production of diacyl glycerol (DAG) and inositol tris-phosphate (IP3), resulting in calcium release from the endoplasmic reticulum (ER). Calcium contributes to many cellular processes including cytoskeletal rearrangement, vesicular transport, and activation of calcium dependent enzymes. Calcineurin, one such calcium dependent enzyme, plays a role in nuclear factor for activated T cells (NFAT) activation and subsequent nuclear translocation where it contributes to the transcription of cytokines such as IL2. PLC γ activation and subsequent DAG formation also leads to the activation of classical and novel PKCs, which play multiple roles in various aspects of activation.

1.4.2 Cytoskeleton Associated Proteins

SLP-76 also serves as a docking molecule for the adaptor protein Nck and for the guanine nucleotide exchange factor Vav-1; substantial evidence suggests that these proteins play important roles in cytoskeletal rearrangement. In T cells, mutations in SLP-76, Vav-1 or Nck result in impaired TCR-stimulated actin polymerization (93) (94) (95). A major function

of Nck in cytoskeletal rearrangement is to facilitate the activation of Wiscott Aldridge Syndrome protein (WASp) (93).

Evidence suggests that WASp activity is linked with Vav-1; defects in Vav-1 or WASp impair actin cap formation and receptor clustering (94) (47) (96). Moreover, in experiments where receptor clustering is contrived using antibody cross linking, T cells isolated from mice deficient in Vav-1 or WASp, show defects in activation and actin polymerization (46) (47) (96). Additionally, inhibiting T cell actin polymerization with cytochalasin D mimics the results of defects in Vav and WASp (47) (95).

The link between Vav-1 and WASp is likely a consequence of the guanine nucleotide exchange function of Vav-1; Vav-1 links TCR stimulation to activation of the Rho-family G-proteins Rac1, Cdc42, and RhoA, all of which have been implicated in cytoskeletal rearrangement (97). Active Cdc42 interacts with WASp and induces an open/active conformation (closed conformation WASp is inert). Open conformation WASp associates with actin related protein 2/3 (Arp2/3), a vital actin regulator that exists in complex with actin related protein 2 and 3 plus 5 other polypeptides (98). The association of WASp with Arp2/3 facilitates Arp2/3 association with actin and corresponding actin polymerization (99).

It is clear that Vav, and WASp-regulated cytoskeletal reorganization and receptor clustering are required for T cell maturation and the induction of physiological T cell responses (43).

1.4.3 PKC

Although Vav-1 and WASp are important for T cell cytoskeletal rearrangement, experiments where cells, deficient in these proteins, are stimulated with PMA show that activation of PKC can bypass the need for both molecules (96) (94) (47). The novel isoform PKC θ is highly expressed in T cells, and cooperates with calcineurin to induce transcription of IL2 (100). Experiments using peripheral T cells from PKC θ knock out mice have shown that PKC θ is important for AP-1 and NFκB activity in peripheral T cells (101). In non-lymphoid mammalian cell systems (i.e. neurons and human erythroleukemia cells), PKC isoforms have been implicated as adaptor proteins involved in linking actin polymerization to specific signaling pathways (102) (103). Considering the evidence that PKC can bypass the need for important cytoskeletal proteins in T cells, and considering that PKC θ colocalizes with the TCR in the immune synapse (104) (58), the candidacy of PKC θ as a critical adaptor between cytoskeletal rearrangement and downstream signaling in T cells is strong. Further supporting evidence suggests that PKC θ is downstream of and dependent on cytoskeletal rearrangement; recruitment of PKC θ to the center of the immune synapse requires Vav-dependent actin polymerization (105). Taken together, this evidence suggests that PKC θ may serve as a link between receptor clustering and SMAC formation, and downstream signaling events.

1.4.4 Paxillin

Paxillin is another cytoskeleton-associated protein, ubiquitously expressed in all cells including T cells, that plays an important role in focal adhesions by serving as an adaptor molecule between structural proteins such as talin, α-actin and vinculin (which anchor the actin cytoskeleton to the cell membrane) and regulatory proteins such as focal adhesion kinase (FAK) (which catalyzes the phosphorylation of other cytoskeleton associated proteins) (106). Paxillin is phosphorylated upon CTL activation (107) (108); paxillin phosphorylation is thought to be important for cell spreading on extracellular matrix (ECM) (109), and for adhesion dependent functions in leukocytes (110). As well, phosphorylation of paxillin facilitates its association with Lck in T cells (107). Taken together, this evidence suggests that the phosphorylation of paxillin is important for cytoskeletal rearrangement as well as for membrane proximal signalling. Moreover, given its dual implications, paxillin may play a crucial role in CTL effector function by bridging cytoskeletal rearrangements and signalling events.

1.4.5 PAK

p21-activated kinase (PAK) is another focal adhesion protein kinase implicated in molecular clustering of the TCR, and actin stabilization. As well, PAK plays a role in mitogen activated protein (MAP) kinase-signalling cascade (111). PAK has traditionally been seen as a downstream effector of Rac1 and Cdc42 and as such, considered dependent on Nck and SLP-76 for activation and relocation. However, PAK has recently been described as a

member of a complex of proteins including pak-interactive exchange factor (Pix β), a recently defined Rac-1 guanine nucleotide exchange factor, Paxillin and paxillin kinase linker (p95PKL). It has been suggested that this newly defined interaction of proteins facilitates the activation of and relocalization of PAK (112). Taken together the evidence suggests that PAK and paxillin interact and as part of a complex, play an important role in cytoskeletal rearrangement and signalling.

1.4.6 Signaling Summary

In summary, receptor engagement on the cell surface initiates signaling events that control the dynamic reorganization of the cytoskeleton, a process that is required for T cell activation and effector function. TCR signaling results in the phosphorylation of the TCR ζ chains resulting in subsequent recruitment and activation of ZAP-70. In turn ZAP-70 phosphorylates LAT and SLP-76, which serve as docking molecules for many downstream proteins including proteins involved in cytokine transcription (i.e. PLCγ and PKC), and cytoskeletal rearrangements (i.e. Nck, Vav-1, WASp, paxillin, PAK). Multiple studies have been done to examine how cytoskeleton-associated proteins interact with actin and induce polymerization; however, many questions remain unanswered regarding how membrane proximal signaling events connect with and signal the recruitment and activation of cytoskeleton-associated proteins, facilitating cytoskeletal rearrangements.

1.5 Phosphatidylinositol 3-kinase

Phosphatidylinositol-3 kinase (PI3K) has been implicated in the initiation of cytoskeletal changes in a number of systems. A focus of this thesis is to determine if PI3K plays a role in cytoskeletal rearrangement required for CTL activation. The discussion of PI3K will be limited to the more relevant aspects including the role of PI3K in sustained signaling, cell spreading, adhesion, immune synapse formation, and MTOC polarization.

1.5.1 PI3K Function

Active PI3K functions as both a serine/threonine kinase and as a lipid kinase. The protein serine/threonine catalytic activity of PI3K varies between isoforms of the p110 subunit (113) but is not well described. In terms of lipid kinase activity, PI3K isoforms share specificity. The lipid kinase activity of PI3K is to catalyze the addition of a phosphate group to position 3 of the inositol ring of its preferred substrate phosphatidylinnositol-4, 5 bis-phosphate (PIP2), resulting in the generation of phosphatidylinnositol-3,4,5- tris phosphate (PIP3) (114) (115). Upon stimulation through the TCR (116), CD28, or through chemokines (117) (118) (119), PI3K is recruited to the membrane where it exerts its lipid kinase activity, resulting in an increase in the membrane content of PIP3 (illustrated in figure 4).

1.5.2 Structure and Regulation.

PI3K exists in many isoforms however; the relevant isoforms to T cell activation are the class I PI3K. Class I PI3Ks are heterodimeric proteins consisting of a p110 α, β, δ or γ catalytic subunit and a p85α, p85β, or p55γ

adapter or linker subunit (120) (121). The p85 subunit is composed of a breakpoint cluster region-homology domain (BH), two SH2 domains, a linker site for the p110-subunit, two proline rich regions, and a src-homology 3 (SH3) domain as illustrated in figure 3 (115) (122) (123). The proline sequences can bind the SH3 domains of Src kinases and other PI3K complexes (124) (125). The BH domain (not shown) binds to Rho-family members Rac1 and Cdc42 while the inter-SH2 domain binds the N-terminal region of p110 (126). The p85 SH2 domain binds phosphorylated YMXM motifs and is thought to be the means by which PI3K is localized to the membrane upon T cell activation.

PI3K activity is regulated by its localization; experimental targeting of the p110 catalytic subunit to the membrane is sufficient to allow for constitutive PI3K activity (127). The adapter subunit does not regulate catalytic activity *per se*, but is important for targeting the catalytic subunit to the membrane where it exerts its enzymatic activity.

1.5.3 Phenotype of PI3K Knock Out

Gene targeting experiments resulting in deletion of p85 α result in reduced B cell development but seem to have little effect on T cells with regard to development or activation; *in vitro* responses to antigen-receptors and costimulation are comparable to wild type (128) (129). However, p85 α knockout (KO) mice retain expression of p85β, which is abundantly expressed in T cells as a complex with p110. Thus p85β likely compensates for the

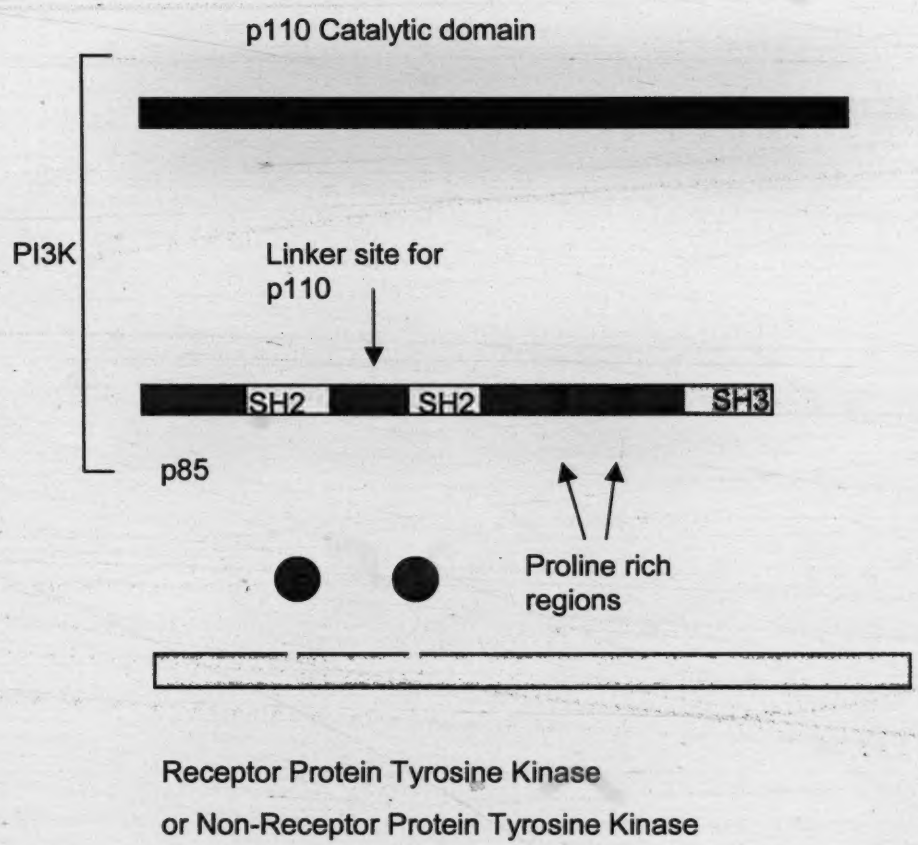


Figure 3. **Structure of Phosphatidylinositol 3-kinase and illustration of domains**

absence of p85 α in T cells. KO mice in p110α have also been made but are embryonic lethal after 10 days gestation (130). Other gene targeting experiments suggest that PI3Kγ is important for thymocyte survival and activation of mature T cells (131). More recently, mice expressing mutant p110δ, generated by Okkenhaug *et al.* were found to have deficiencies in antigen receptor signaling in both T and B cells (132). As well immune responses in these mutant mice are attenuated *in vivo*. These studies clearly illustrate that PI3K is important for T cell responses to various stimuli leading to numerous biologically significant outcomes.

1.5.4 PI3K Activation: Recruitment to the Membrane

The first models of PI3K activation in T cells were based on previously established models in non-haematopoietic cells. In this context, PI3K is thought to be recruited to the membrane in response to CD28 signalling; CD28 interaction with B7-1/2 results in the phosphorylation of the YMNM cytoplasmic motif of CD28 allowing association of the SH2 domains of the p85 adaptor subunit (117). Another model for signalling PI3K recruitment is through TCR signalling (133). This model involves association of PI3K with an ill-defined phosphorylated adaptor molecule, current contenders are LAT and TRIM as illustrated in figure 4 (73) (134). Both models of PI3K relocalization rely on similar rules, the SH2 domain of p85 likely interacts with a newly phosphorylated membrane-associated molecule in response to receptor signalling.

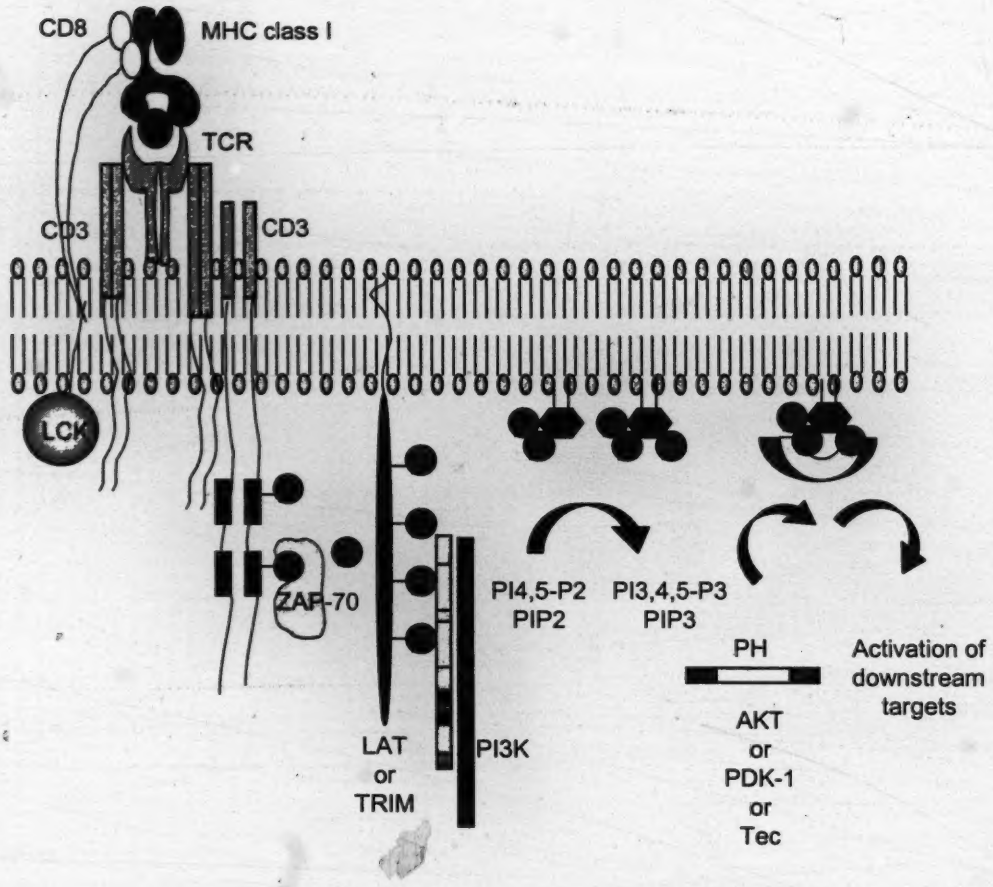


Figure 4. **PI3K Activation in Response to TCR signaling.**
TCR engagement with MHC class I and agonist peptide induces a signaling cascade whereby PI3-Kinase is recruited to the membrane through interaction with an adaptor molecules. At the membrane, PI3K converts PIP2 to PIP3. PIP3 acts as a docking site for proteins containing pleckstrin homology domains such as AKT.

Ongoing research addresses the role of PI3K in CD28, and TCR-mediated signaling. Although studies addressing TCR signaling show conclusively that PI3K is activated and functions in multiple signaling cascades (see below), studies with CD28 have conflicting results and are highly controversial, thus no consensus has been reached as to whether or not PI3K is activated in response to CD28 signaling.

1.5.5 Downstream Targets of PI3K

One product of PI3K, PIP3, localizes to lipid rafts (135) and can serve as a docking molecule for proteins containing pleckstrin homology (PH) domains (115). This interaction facilitates the relocalization of PH domain containing proteins to the membrane, where they can become activated and/or have access to their downstream targets (see figure 4). Binding PIP3 in the membrane may also induce conformational changes that allow allosteric modifications of activity (136).

1.5.6 Protein Kinases

PI3K activity is required for the localization and activation of many protein kinases. One family of major importance includes the Tec family kinases. These tyrosine kinases are important for activation of PLC γ, discussed previously. The critical nature of the PH domain interaction between Tec family kinases and PIP3 is illustrated by mutations in the PH domain of Brutons tyrosine kinase (Btk: a Tec kinase family member), which results in X-linked agammaglobulinaemia in humans and a similar immunodeficiency in mice (137).

PIP3 binding other proteins such as the serine/threonine kinases phosphatidylinositol dependent kinase-1 (PDK-1) and protein kinase B (PKB or AKT) through PH domains allows for their recruitment to the membrane and subsequent phosphorylation and activation (115) (121).

1.5.7 Cytoskeletal Interactions

PI3K has been implicated in the activity of Rac-1, Rho and Cdc42, which contribute to the dynamic organization of the actin cytoskeleton and the assembly of associated integrin structures (127) (138) (139). The Rac-1 guanine nucleotide exchange factor (GEF), Vav-1 contains a PH domain, and has been shown to associate with PIP3, an interaction that promotes tyrosine kinase mediated activation of this enzyme (140). PIP3 generated by PI3K, also contributes to the activation of WASp as a result of PIP3 interaction with the PH domain of WASp (141).

PI3K has been implicated in the relocalization of other cytoskeleton associated protein complexes. Pix β, a Rac-1 guanine nucleotide exchange factor first identified in 1998, contains a PH domain (142) (143). Moreover, Yoshii *et al* have found evidence to suggest that the activation of Pix β in rat brain extracts and HeLa cells is dependent on PI3K (144). It is plausible that the PH domain of Pix plays a role in localization of the PAK/Paxillin/p95PKL/Pix complex to the membrane upon T cell activation, however that this complex actually forms in T cells is currently an object of debate; there is no association of the complex with paxillin in Jurkat T cells (112). As well, if the complex forms in T cells, it remains to be elucidated if

localization is PI3K dependent, and if so, what the downstream effects are on the molecules involved in the complex.

1.5.8 Adhesion and Cytoskeletal changes

PI3K has been implicated in the cell spreading of numerous cell types. Lowell and colleagues have shown that blocking PI3K activity inhibits macrophage spreading on fibronectin (145). Similarly, treatment of platelets with PI3K inhibitors results in inhibition of normal actin cytoskeletal rearrangements (146). PI3K clearly plays a role in cell spreading in other leukocytes, however, a clear role for PI3K in T cells remains to be elucidated.

CTL conjugation is mediated by LFA-1, and to a lesser extent, by CD8. In T lymphocytes, signalling through a variety of receptors including the T cell receptor and CD28 results in the induction of integrin activation (147), (148), (149) and, increases the affinity of CD8 for class I MHC (41). At the same time, signalling through these receptors results in the recruitment and activation of PI3K at the membrane. It has thus been postulated that PI3K participates in signalling events that mediate inside out signalling, resulting in integrin activation and CD8 affinity enhancement (150) (151).

A number of studies support a role for PI3K in adhesion. King *et al*, have shown that plating fibroblasts on fibronectin-coated surfaces causes the clustering of surface integrins, leading to enhanced PI3K activation (152) suggesting that adhesion initiates PI3K signalling. More recently, studies on cytohesin-1, the cytoplasmic regulator of β2 integrin adhesion to ICAM-1, have shown that cytohesin-1 recruitment to the cytoplasmic domain of LFA-1

is PI3K dependent (151). Cytohesin-1 contains a PH domain that specifically interacts with PIP3 in the membrane facilitating not only compartmentalization for cytohesin-1, but also contributing to the specificity of the GEF activity of cytohesin-1 (151), (153), (154). These data suggest that PI3K is an upstream regulator of cytohesin-1 and thus, may contribute to inside out signalling and cell/cell adhesion. Also, Hydek *et al* have shown that cross-linking either α-4 integrins or the CD3 on Jurkat cells induces adhesion to ICAM-1 in a PI3K dependent manner (155) however; their study does not support a specific mechanism for PI3K involvement.

Mescher and colleagues add to the evidence supporting a role for PI3K in adhesion, however, their evidence suggests that PI3K plays a role in the signalling events that initiate with TCR stimulation and result in the up-regulation of CD8 dependent adhesion (156). As well, PI3K has been implicated in the inside out signalling of β1 integrins (157). Taken together, these results strongly suggest that PI3K is a ubiquitous regulator of triggered binding and adhesion required for various receptor systems.

1.5.9 Summary

PI3K is a heterodimeric protein that functions to convert PIP2 to PIP3 in the membrane. PIP3 functions as a docking molecule for cytoplasmic proteins that contain PH domains and has been implicated in cytoskeletal rearrangements, T cell activation and degranulation. The exact role of PI3K in T cell activation and cytoskeletal rearrangement remains to be elucidated.

1.6 Hypothesis to be tested

- Given that PI3K has been implicated in specific signalling cascades including TEC kinase signalling, and given the implication of PI3K activity in cytoskeletal rearrangements in other cell types, we hypothesize that PI3K plays a role in membrane proximal signalling events that contribute to cytoskeletal reorganization.

Chapter II

Materials and Methods

2.1 Cell lines

The CTL clone AB.1 (murine $H2^d$ anti-$H2^b$) which have been described previously (158), were grown in HEPES-buffered RPMI supplemented with 10% heat inactivated fetal bovine serum, Sodium Pyruvate, non-essential amino acids, L-glutamine, penicillin-streptomycin, and β-2 mercaptoethanol. Cells were stimulated weekly with irradiated C57BL/6J spleen cells in media supplemented with IL2 and experiments were performed 4 to 6 days after stimulation.

The L1210 lymphoma cell line with chimeric class I MHC was a generous gift from Dr. Kevin Kane (University of Alberta). Briefly, L1210 cells were transfected with class I containing the α1/α2 domains from K^b and the α3 domain from D^d (M, Durairaj and K. Kane, manuscript in preparation). L1210 cells were grown in DMEM supplemented with 8% heat inactivated defined calf serum (DCS).

2.2 Antibodies

Monoclonal antibodies 145-2C11 (α-CD3) and M17/5.2 (α-LFA-1) were obtained through American Type Culture Collection (ATCC) (Manassas, VA). The monoclonal antibodies for paxillin and p95 PKL were purchased from Transduction Laboratories (Lexington, KY) and Vav, from Upstate

Biotechnology (Lake Placid, NY). CD45 RB monoclonal antibodies MB4B4 and MB23G2 were used as culture supernatants from hybridomas purchased from ATCC (Manassas, VA). PY72 (α-phospho-tyrosine) was obtained from Dr. B. Sefton (Salk Institute, La Jolla CA). Hybridomas were grown in Protein Free Hybridoma Medium-II (Gibco- BRL, Burlington, ON). The monoclonal antibodies were purified by ammonium sulfate precipitation and, when necessary, further purified by protein A or protein G chromatography. Fibronectin used for costimulation (10 μg/ml) was purchased from Upstate Biotechnology, Inc., (Lake placid NY), and anti-CD28, also used for costimulation (10 μg/ml) from BD Pharmingen. Polyclonal antibodies specific for AKT and for phospho-AKT (Ser 473) were obtained from Cell Signalling Technology (Beverly, MA). The p110 antibody (α-PI3K) was purchased from Santa Cruz Biotechnology, (Hornby, Ontario). PKC θ antiserum was provided by Dr. C. Arendt (Skirball Institute, NY). Goat α-mouse PKC theta antibody C-18 and α-goat^{HRP} antibody were purchased from Santa Cruz Biotechnology. Hamster α-mouse phospho-p44/42 ERK (Thr202/Tyr204) antibody 9101 was purchased from New England Biolabs (Beverly, MA). Rabbit α-hamster^{HRP} and α-mouse^{HRP} were purchased from Jackson Immunoresearch Laboratories (West Grove, PA), and protein A^{HRP} from Pierce (Brockville, Ontario).

2.3 Chemical Reagents and Inhibitors

Wortmannin, the fungal metabolite used to irreversibly inhibit PI3K activity was purchased from Calbiochem (San Diego, CA), and used at concentrations of 0.1 to 100 nM. LY 294002 was also purchased from Calbiochem and used at concentrations of 0.01 μM to 50μM. CTL were pretreated with LY 294002 and wortmannin for 30 minutes at 37°C prior to stimulation. Depending on the experiment, wortmannin was either left in culture, or washed out. Ionomycin (used at 2μM) was purchased from Calbiochem (San Diego, CA) and TPA (PMA) (used at 150ng/ml) was purchased from Sigma Chemicals (Mississauga, Ontario). Cell viability was checked by trypan blue exclusion. Neither of the drugs used had a significant impact on cell viability over the duration of the assays.

Cytochalasin E, used at 10 μM to disrupt actin filaments after 15 minutes of stimulation, was purchased from Sigma Chemicals (San Diego, CA). Alkaline Phosphatase, purchased from Promega (Madison, WI) was used to cleave phosphate residues from immunoprecipitated protein. After washing, immunoprecipitated protein was resuspended in mQ H_2O 100 μl + 10 μl buffer + 20 μl Alkaline phosphatase.

2.4 Cell Stimulation

AB.1 cells were stimulated by one of three methods. For immobilized anti-CD3 monoclonal antibody stimulation, ninety-six well flat bottom Falcon 3912 microtitre plates (Becton Dickinson, Oxnard, CA) or 60 x 15 mm, non-

treated polystyrene dishes (Fischer Brand Extra Deep Dishes/Fischer; Nepean, Ontario) were coated with 10 μg/ml 145-2C11 anti-CD3 overnight at 4°C. Wells were washed three times with phosphate buffered saline (PBS), blocked with 2% bovine serum albumen (BSA) in PBS for 30-60 minutes at 37°C, then washed three times with PBS before use. For phorbol ester plus calcium ionophore stimulation, 150 ng/ml of phorbol-12-myristate acetate (PMA or TPA) was added together with 2μM ionomycin to AB.1 CTL in D-PBS, and cells were immediately aliquoted to BSA-blocked plates or dishes as described above. Some experiments required PMA alone, for those experiments PMA was added to AB.1 at 100ng/ml. For target cell stimulation, cells in D-PBS were cocultured at a 1:2 or a 2:3 effector to target ratio at 4°C followed by a cold spin (4°C for 3 minutes at 700-800 RPM). The cocultures were then incubated at 37°C to initiate stimulation.

2.5 Degranulation Assay

Degranulation, as measured by the release of serine esterase, was assayed as previously described (159). AB.1 cells were washed 3X by centrifugation in PBS and resuspended in 2% newborn calf serum in RPMI. AB.1 were then stimulated by addition at 1.5 x 10^5 cells/well directly to wells of 96 well plates previously coated with 145-2C11 (anti-CD3 antibody), or mixed at a 2:3 with target cells in a total volume of 150 μl, or stimulated with 150 ng/ml TPA and 2 μM Ionomycin. Plates were then incubated at 37°C for 4.5 hours after which 25 μl supernatant was assayed for Benzyloxy carbonyl-

L-lysine Thiobenzyl (BLT)-esterase activity (159) and read in a kinetic micro plate reader (Molecular Devices) at 405 nm, after approximately 20 minutes. All conditions were performed in triplicate and the standard deviation calculated. All experiments were done at least three times with similar results.

2.6 SDS-PAGE, Immunoblotting and Immunoprecipitation

For assays involving CTL stimulated with anti-CD3 antibody, 1.5 $x10^5$ CTL in 40 μl D-PBS containing phenol red (Gibco-BRL, Burlingtom, ON) were directly added to antibody-coated wells. After incubation at 37°C for the indicated times, the cells were lysed by addition of 40 μl 2X Laemmli reducing sample buffer (RSB) and the lysates separated on 7.5 or 8.5% sodium dodecyl sulfate polyacrylamide gel elecrophoresis (SDS-PAGE) gels (for MAP kinase mobility shift assays, a 15% low-Bis (175:1 acrylamide:Bis) gel was used. The activated forms of p44Erk1 and p42Erk2 experience reduced mobility under these conditions). Proteins were transferred to immobilon P (Millipore Corporation, Bedford, MA) and immuno-blotted using various primary antibodies. Secondary antibodies coupled to horseradish peroxidase (α-mouse^{HRP}, α-goat^{HRP} or protein-A$^{HRP)}$ were used to visualize the proteins by an enhanced chemiluminescence (ECC) system (NEN Life Science Products, Boston, MA).

For assays involving CTL stimulated with target cells, CTL and target cells were washed as previously stated and resuspended in D-PBS

containing phenol red at concentrations of $2.5x10^6$ and $5x10^6$cells/ml respectively. Cells were cocultured on ice and centrifuged to optimize interaction for 4 minutes at 700 RPM. Conjugate pellets were then incubated at 37°C for various times, after which the supernatant was aspirated and replaced with lysis buffer (1.0% NP40, 20 mM Tris (pH 7.4), 150 mM NaCl, 5mM ethylenediaminetetraacetate (EDTA), and 1% vanadate), the cells were resuspended in 40μl and kept on ice. The nuclei were removed by centrifugation at 4°C for 2-3 minutes at 200 RPM. Post nuclear lysates were then combined with 40 μl 2X Laemmli reducing sample buffer, boiled and the proteins separated by SDS page. The blotting procedure is as above.

Paxillin, Vav, and PKC θ were immunoprecipitated (IPed) by adding 10^7 CTL to 145-2C11-coated 5 cm dishes. After incubation at 37°C for 20 minutes the cells were lysed in 1.0% NP40, 20 mM Tris (pH 7.4), 150 mM NaCl, 5mM EDTA, and 1% vanadate and gently agitated for 20 minutes at 4°C. 40 μl of pre-nuclear supernatant was reserved and used as lysate samples. The nuclei were removed by centrifugation at 16,000x g for 5 minutes. After clarification of the lysates, primary antibody specific for paxillin, Vav, or PKC θ was added to each sample, and the lysates incubated on ice for 15 minutes followed by the addition of the appropriate secondary antibody for 15 minutes. Protein A sepharose beads were then added and the samples incubated at 4°C for 2 hours on a rotator. The beads were then washed 3X by centrifugation in 1% NP40 in PBS and then two additional times in PBS. Proteins were eluted from the beads using 1X Laemmli

reducing sample buffer and the denatured protein separated on 8.5% gels and blotted as above.

2.7 Conjugate Assay

CTL and target cells were washed with PBS and stained with green PKH67 and red PKH26 lipophilic dyes respectively (Sigma Chemicals, St. Louis, MO). The cells were then washed 3X in 5% DCS PBS, and incubated at 37°C for a 1-2 hour rest period to prevent dye transfer. During the rest period (where indicated), CTL were treated with 50 nM wortmannin for 30 minutes followed by 1 wash in 5% DCS in PBS. Cells were resuspended at $1x10^6$ cells/ml (effectors) and $2x10^6$ cells/ml (targets) in 5% DCS in PBS. The cells were then cocultured at 4°C by adding 100 μl of CTL and 100μl of targets to FACs tubes for a total of 200 μl. The cocultures were centrifuged at 4°C at 800 RPM for 3 minutes. At time zero cocultures were incubated in a 37°C water bath for the times stated followed by gentle vortexing and fixation in 200 μl of 4% paraformaldyhyde. Conjugate formation was measured by Flow Cytometric analysis; conjugates were defined as pairs of cells fluorescing both red and green. The percent conjugation was measured by dividing the number of AB.1 conjugates/total number of AB.1.

2.8 MTOC polarization

CTL and target cells were washed as above. Target cell membranes were counterstained with florescence isothyocyanate (FITC) (Molecular

Probes, Hornby, ONT). Targets and CTL were then cocultured at 4 C by centrifugation at 700 RPM followed by incubation at 37 C. After 20 minutes the cells were allowed to adhere to poly-l-lysine cover slips (BD Labware, Two Oak Park, MA) for 5-7 minutes followed by fixation and solublization with cytofix/cytoperm (BD Pharmingen). Slips were washed 2X in perm/wash buffer (BD Pharmingen) and allowed to block for 30 minutes at room temperature followed by addition of Percentrin antibody (Covance, Richmond, CA) specific for the MTOC, and then secondary ALEXA^{594} (Molecular Probes, Hornby, ONT). Excess antibody was washed 2X, using perm/wash buffer, and the slips were fixed in place on slides. MTOC polarization was detected using confocal microscopy. CTL and target cells were identified as conjugated if the CTL membrane adsorbed to the target. The CTL was then sectioned visually into thirds and the position of the MTOC recorded. The MTOC was counted as polarized if it was located within the 1/3 closest to the target.

2.9 Cell Fractionation

$5x10^7$ AB.1 were cocultured with $7.5x10^7$ target cells on ice. The cells were loosely pelleted by cold spin at 700 RPM for 3 minutes. The pellets were then incubated at 37°C for the indicated times, the supernatant was aspirated and the pellets resuspended in ice-cold Dounce buffer (10mM Tris/HCl ph 7.6, 0.5mM $MgCl_2$, 1mM phenylmethyl sulfonyl fluoride (PMSF)) for 10 minutes. Then the mixtures were homogenized, and the tonicity

restored by the addition of 0.6 M NaCl to a 0.15 M final concentration. 40 μl samples of the mixtures, taken at this point and denatured by 2X RSB, were designated as crude. The nuclei were then removed during a cold spin at 500x g for 5 minutes. The post-nuclear supernatant was then treated with EDTA to a 5mM final concentration and the mixtures centrifuged at 100,000x g at 4°C for 45-60 minutes to separate the soluble from the insoluble fractions. The soluble fraction (cytoplasm) was removed as the supernatant and denatured by addition of 2X RSB and the insoluble fraction (membrane + cytoskeleton) resuspended in 0.1% Triton X 100 and allowed to solublize at 4°C for 30-45 minutes. In some experiments, membrane and cytoskeleton were separated by a cold spin at 10 000 x g for 15 minutes. In others, membrane and cytoskeleton were kept together. Samples of each fraction were taken and denatured by addition of 2X RSB.

Denatured fractions were separated by 8.5% SDS-PAGE, transferred to immobilon-p and immunoblotted using antibodies specific for phospho-tyrosine, PKC θ, CD45 RB, and paxillin and their corresponding HRP-conjugated secondary antibodies.

Chapter III

PI3K activity is required for Paxillin Phosphorylation and CTL Adhesion.

3.1 Introduction

PI3K is a lipid kinase that has been implicated in multiple cellular processes. Previous studies have shown that PI3K is important for cell spreading on fibronectin (160) (145) implying a role for PI3K in cytoskeletal rearrangements. Others have shown that PI3K activity is required for CTL degranulation (161), indirectly suggesting that cytoskeletal rearrangements are important for effector function. Our lab has found evidence supporting that in fact cytoskeletal rearrangements are required for lymphocyte effector function. Our previous studies have shown that CTL spreading is absolutely required for sustained tyrosine phosphorylation of signaling proteins, which facilitates degranulation; cross-linking the TCR as opposed to stimulating the TCR with immobilized anti-CD3, does not result in sustained tyrosine phosphorylation, nor does it stimulate CTL to degranulate (61)

Although cytoskeletal rearrangements are required, little is known about how TCR initiated signaling events trigger cytoskeletal rearrangement within the cell. The objective for this study involved exploring this question, and determining the candidacy of PI3K as such a player. We hypothesized that if PI3K plays a role in cytoskeletal rearrangements in CTL, then perhaps it could be a potential link between membrane proximal signaling events and

cytoskeletal rearrangement, and as such would be required for CTL effector function.

3.2 PI3K inhibitors inhibit the phosphorylation of AKT, a protein dependent on PI3K activity for its phosphorylation.

In order to test the hypothesis that PI3K is important for cellular processes in CTL clones, we sought out a method to inhibit the activity of PI3K. The two commercially available drug inhibitors of PI3K activity are wortmannin and Ly 294002. Wortmannin, a cell permeable fungal metabolite, acts as a selective, irreversible inhibitor of the catalytic domain of PI3K. Ly 294002, another cell permeable inhibitor of PI3K activity acts reversibly on the ATP binding site of PI3K. Although Ly 294002 is arguably more specific for PI3K then wortmannin, the irreversible nature of wortmannin makes it more useful for experiments involving live cells, and for addressing the effects of completely blocking the activity of PI3K. Moreover, wortmannin is highly specific at concentrations less then 500 nM (162); in the experiments outlined in this thesis, wortmannin is used at concentrations no higher then 100 nM. Thus, to inhibit PI3K activity in CTL we employed the use of both inhibitors for some experiments and selectively used wortmannin for others.

Upon CTL activation, the Ser/Thr kinase AKT, also known as PKB, is recruited to the membrane as a result of interaction between PIP3 with the PH domain of AKT. At the membrane, AKT is phosphorylated by PDK-1 in a PI3K dependent manner and can in turn act on downstream substrates (e.g.

Bad and glycogen synthase kinase GSK). We wanted to ensure that wortmannin and Ly treatment do in fact inhibit PI3K activity and thus, the phosphorylation of AKT. Figure 5A and figure 5B (top panels) show that in CTL, AKT is phosphorylated upon stimulation with immobilized anti-CD3 antibody, and that treatment with wortmannin or with Ly 294002 inhibits the phosphorylation of AKT in a concentration dependent manner. Figure 5A and 5B (Bottom panels) show that AKT is present in an unphosphorylated state in all samples. These data support that wortmannin and Ly 294002 treatment are effective inhibitors of PI3K activity as measured through the phosphorylation of downstream substrates.

3.3 PI3K activity is required for CTL degranulation.

A key question that we wanted to address was whether PI3K is required for effector function as measured by degranulation. To answer this question, the CTL clone AB.1 was pre-treated with various concentrations of wortmannin or Ly 294002 to inhibit PI3K activity, and then stimulated with immobilized anti-CD3 antibody. After 4.5 hours, degranulation was measured by sampling the supernatant for serine esterase release. Figure (6A) and figure (6B) show that CTL degranulation is inhibited in a concentration dependent manner.

To extend this observation, we also stimulated CTL with antigen bearing target cells (L1210 $^{Kb/Dd}$) and found that degranulation was inhibited to the same extent as when CTL were stimulated with anti-CD3 (6A.). The

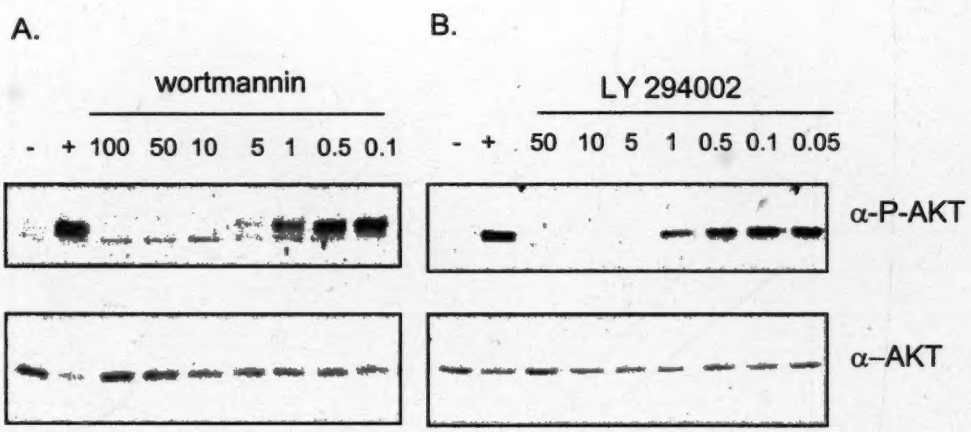


Figure 5. **Wortmannin and LY 294002 treatment inhibit PI3K activity as shown through the phosphorylation of AKT.** AB.1 CTL clones were pretreated with wortmannin (A) or with Ly 294002 (B) at the indicated nM (wortmannin) or μM (Ly) concentration for 30 minutes at 37°C before stimulation with immobilized 145-2C11(α-CD3) for 20 minutes. Whole cell lysates were blotted using phospho-AKT antibodies (top panel) and AKT antibodies (bottom panel). (-) indicates basal conditions with no no drug treatment. (+) indicates stimulation but no drug treatment. This experiment was repeated 3 times with similar results.

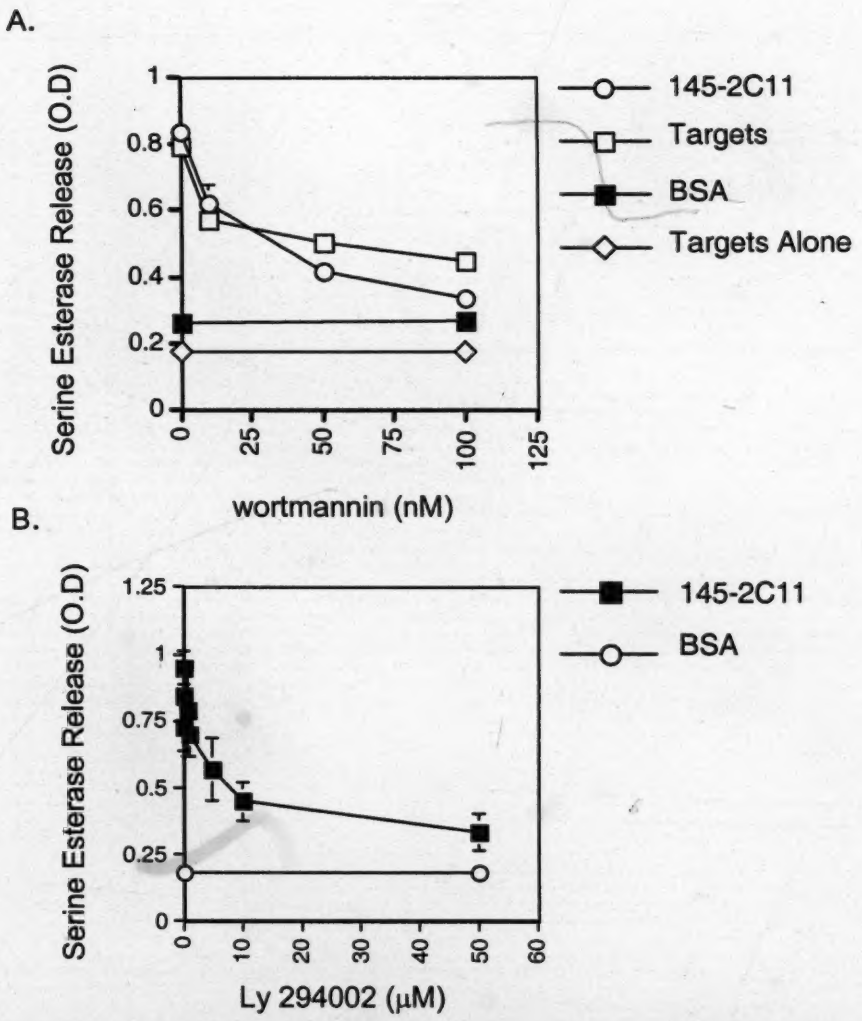


Figure 6. **PI3K activity is required for optimal CTL degranulation.** AB.1 CTL clones were pretreated with wortmannin (A) or with Ly 294002 (B) for 30 minutes at 37°C at the indicated concentrations, prior to stimulation with immobilized 145-2C11 (α-CD3) or with target cells. Target cells bear the relevant MHC (L1210 K^b/D^d). Stimulation lasted for 4.5 hours, after which the supernatants were collected and assayed for serine esterase as an indicator of degranulation. ((A) Unstimulated AB.1 (-■-), AB.1 stimulated with immobilized 145-2C11 (-O-), or L1210 $^{Kb/Dd}$ target cells (-□-). As a control, L1210 $^{Kb/Dd}$ target cells were cultured alone (-◇-).) ((B) (-■-) AB.1, pretreated with Ly294002 and stimulated with 145-2C11, (-O-) AB.1 left unstimulated.)

data in figure 6A represent one of three identical experiments where AB.1 were stimulated with target cells bearing the appropriate antigen and wortmannin was used to inhibit PI3K activity. Similar results were found when PI3K was inhibited using Ly 294002 (Appendix Figure 1). These results suggest that PI3K is a critical player in the intracellular events that lead to CTL degranulation.

3.4 PI3K is required for signaling events upstream of the process of degranulation.

Since we, and others have shown that PI3K activity is required for degranulation, we sought to determine if PI3K is required for early or late events leading to degranulation. Therefore we examined the effects of PI3K inhibitors on CTL stimulated to degranulate by a diacylglycerol analogue (TPA) together with a calcium ionophore (Ionomycin) thus bypassing membrane proximal signalling events. Figure 7 compares the effects of wortmannin treatment on CTL stimulated with TPA/Ionomycin to CTL stimulated with immobilized anti-CD3. This figure, which represents one of three similar experiments, demonstrates that degranulation is inhibited in wortmannin treated cells stimulated with anti-CD3, but not when CTL are stimulated with TPA/Ionomycin. This suggests that PI3K is not required for the process of degranulation, but is likely required for the signalling events leading to degranulation by CTL.

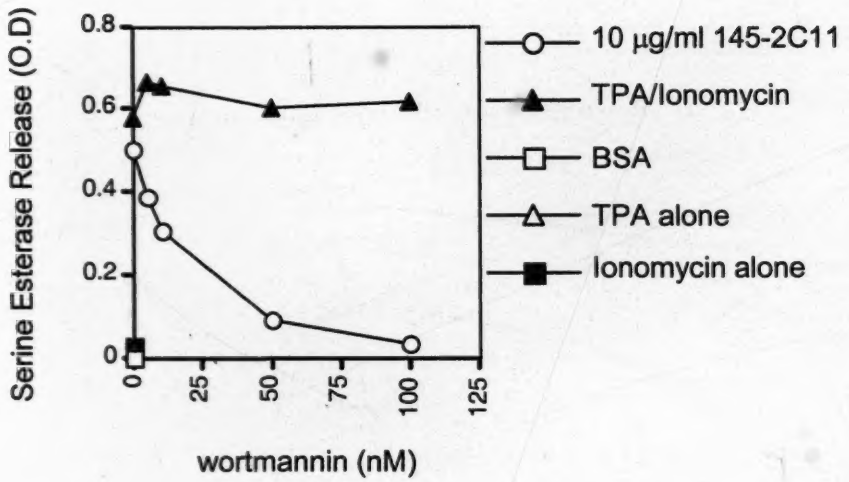


Figure 7. **PI3K is required upstream of the process of degranulation**. AB.1 CTL clones were pretreated with varying concentrations of wortmannin for 30 minutes at 37°C followed by stimulation with 145-2C11, or 150ng/ml TPA + 2µM Ionomycin. After 4.5 hours the supernatants were sampled and assayed for serine esterase release. (-O- AB.1 stimulated with 145-2C11, -▲- AB.1 stimulated with TPA/Ionomycin, -Δ- AB.1 stimulated with TPA alone, -■- AB.1 stimulated with Ionomycin alone, -□- unstimulated AB.1.)

3.5 PI3K plays a specific role in the tyrosine phosphorylation of membrane proximal proteins upon CTL stimulation.

Because PI3K is important for signaling leading to degranulation of CTL, we set out to determine if inhibiting PI3K affects the tyrosine phosphorylation of proteins involved in early signaling events. In general western blots of whole cell lysates from CTL, pretreated with wortmannin or with Ly 294002, showed that inhibiting PI3K activity had little effect on the tyrosine phosphorylation of membrane proximal proteins (Figure 8A and B Top panel) suggesting, that most early tyrosine phosphorylation pathways are not dependent on PI3K. Interestingly, we found that in the region between 58 and 84 KDa, tyrosine-phosphorylation is specifically inhibited. From our previous studies on paxillin, we recognized that this is the position of the gel where phosphorylated paxillin migrates. We therefore probed the blots with antibody specific for paxillin and found that the shift of paxillin from a faster to a slower migrating species is inhibited with wortmannin or Ly 294002 treatment (Fig 8A and B, middle panel). Figure 8A and B (bottom panel) shows a VAV blot as a loading control. The results shown are representative of three similar experiments.

3.6 PI3K plays a role in the phosphorylation of Paxillin upon CTL activation.

In order to show that the shift in paxillin from a lower molecular weight form to a higher molecular weight form was in fact due to phosphorylation, and that the shift is inhibited by wortmannin treatment, paxillin was

A.

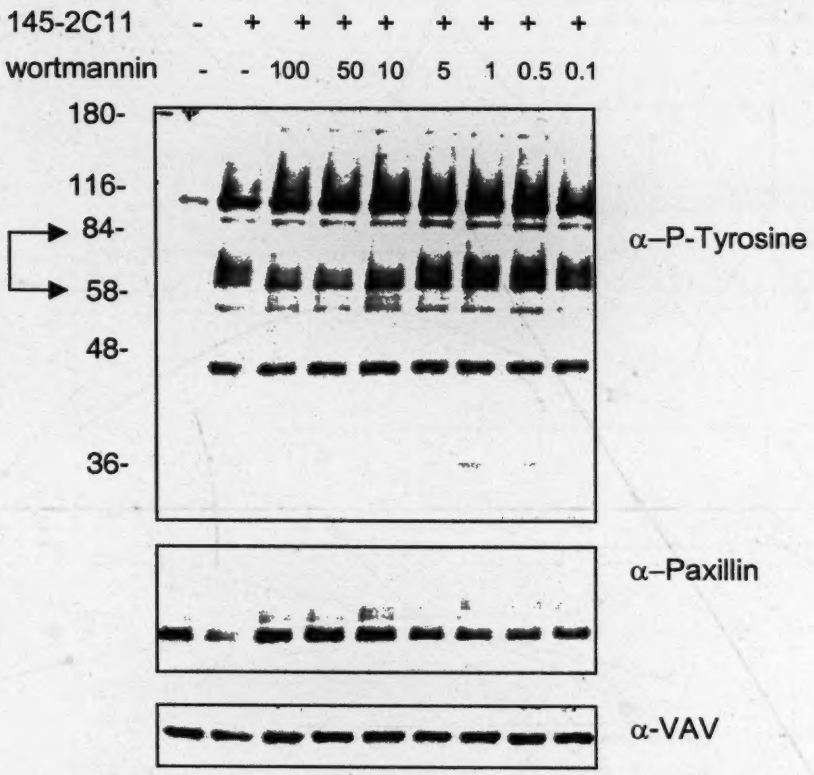


Figure 8. A. **PI3K activity is required for phosphorylation of a specific subset of proteins**. A. AB.1 CTL clones were pretreated with wortmannin at the indicated nM concentrations for 30 minutes at 37°C followed by stimulation with immobilized 145-2C11 for 20 minutes. Reduced whole cell lysates were blotted with phospho-tyrosine antibody, (top panel), paxillin antibody (middle panel) and Vav antibody (bottom panel).

B.

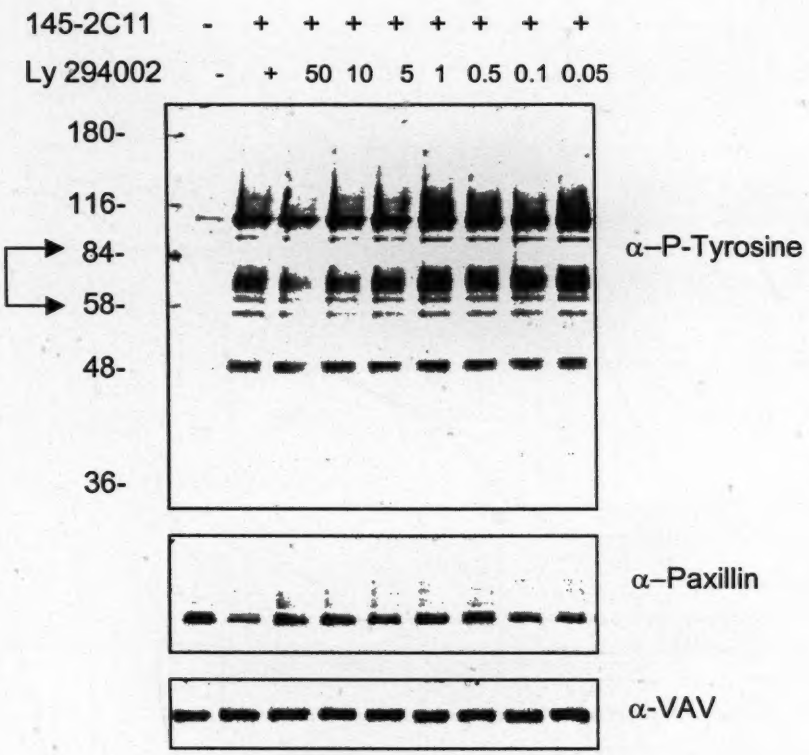


Figure 8. B. AB.1 CTL clones were pretreated with Ly 294002 at the indicated μM concentrations for 30 minutes at 37°C followed by stimulation with immobilized 145-2C11 for 20 minutes. Reduced whole cell lysates were blotted using phospho-tyrosine antibody (top panel), paxillin antibody (middle panel) and Vav antibody (bottom panel).

immunoprecipitated from CTL pre-treated with wortmannin and stimulated with immobilized anti-CD3 antibody. Figure 9 (Top panel) shows that paxillin is highly tyrosine phosphorylated upon anti-CD3 stimulation and that this phosphorylation is partially inhibited with wortmannin treatment at 50 and 10 nM. However, wortmannin significantly reduces the mobility shift, which has contributions from Ser/Thr phosphorylation. Alkaline phosphatase treatment confirms that the shift is due to phosphorylation (data not shown). Figure 9 (Middle panel) is a control showing that the protein present from the IP is in fact paxillin.

Because PI3K has a dramatic effect on paxillin phosphorylation, we wanted to address if this effect was direct, through a PI3K/paxillin interaction, or indirect, as a result of other protein interactions and or PIP3 in the membrane. Thus figure 9 (Bottom panel) shows the result of a Paxillin IP stripped and re-probed with antibody specific for the catalytic domain of PI3K. Under the conditions used for the IP, we cannot detect a PI3K/Paxillin complex. The same is true for AKT; a paxillin/AKT physical interaction is not detectable in IPs from AB.1 CTL stimulated with immobilized anti-CD3 (data not shown).

Different proteins involved in CTL activation, become phosphorylated at specific times during activation. We next wanted to address the kinetics of paxillin phosphorylation; when it occurs and how long is it maintained during CTL activation. As well we wanted to address whether inhibiting PI3K had an

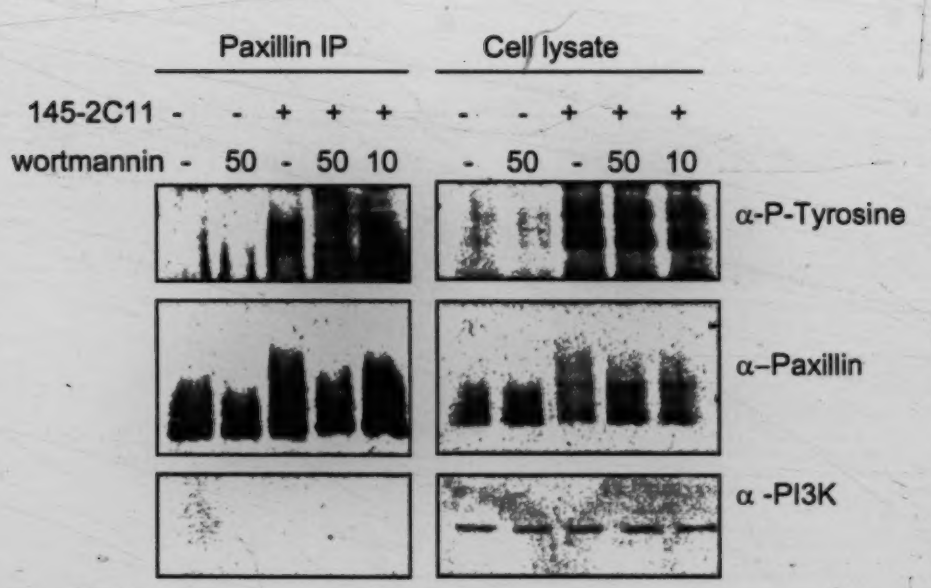


Figure 9. **PI3K activity is required for the Phosphorylation of Paxillin.** AB.1 CTL clones were pretreated with 50 nM and 10 nM wortmannin, for 30 minutes at 37°C followed by stimulation with immobilized 145-2C11 for 25 minutes. Reduced paxillin immunoprecipitates and whole cell lysates were blotted with phospho-tyrosine antibody (top panel), paxillin antibody (middle panel), and PI3K antibody (bottom panel). This experiment was repeated 5 times with similar results.

effect on initiating the phosphorylation of paxillin, or in maintaining the phosphorylation of paxillin. Figure 10 shows the results of a kinetic assay addressing these questions wherein CTL were either pre-treated with 50 nM wortmannin or left untreated, and stimulated with immobilized anti-CD3 for the indicated times before being lysed under reducing conditions. Figure 10 represents one of three experiments, figure 10A shows a control where the lysates were probed with antibodies specific for tyrosine-phosphorylated proteins and shows that tyrosine phosphorylation increases over time. Figure 10B shows that Paxillin becomes heavily phosphorylated after approximately 20 minutes (top panel) of stimulation with anti-CD3. This form remains unaltered throughout a 60-minute time course of stimulation. Wortmannin treatment impedes the formation of this higher MW form all together and maintains paxillin in a low MW, unphosphorylated form (bottom panel). Taken together these results show that TCR stimulation triggers the phosphorylation of paxillin over time, and that wortmannin treatment inhibits phosphorylation of paxillin.

To extend our observation to CTL stimulated with target cells, we performed an assay whereby CTL were either treated with wortmannin or left untreated and then stimulated through coculture with target cells that do or do not bear the appropriate antigen. Western blot analysis of whole cell lysates shows tyrosine phosphorylation of both CTL and target cell proteins upon coculture (figure 11A). Figure 11B shows that the paxillin shift, likely triggered in both CTL and targets upon coculture, is inhibited with wortmannin

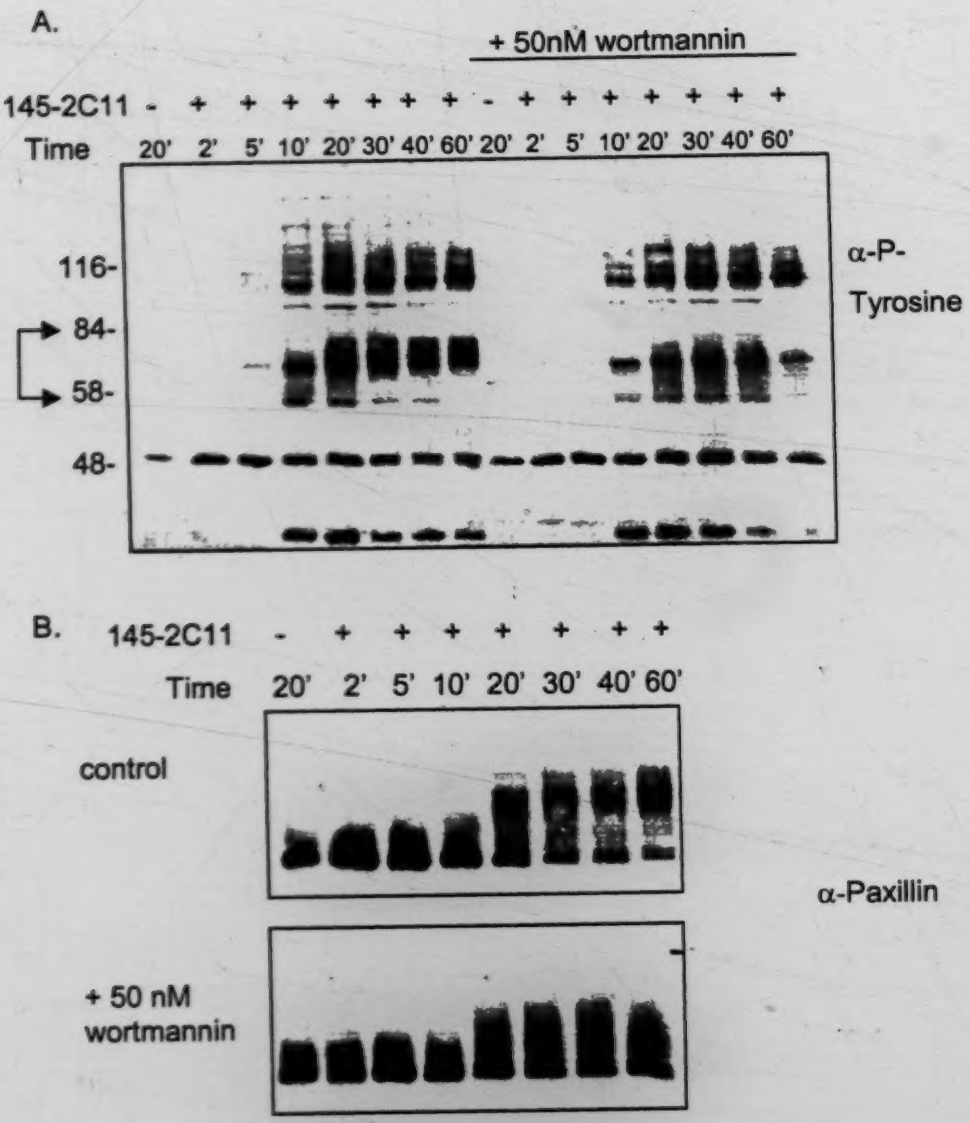


Figure 10. **Paxillin Phosphorylation, stimulated by immobilized α-CD3, requires PI3K activity.** AB.1 CTL clones, pretreated with 50 nM wortmannin, or left untreated, were stimulated with 145-2C11 over a time course. Reduced whole cell lysates were blotted using antibodies specific for phospho-tyrosine (A) and paxillin (B).

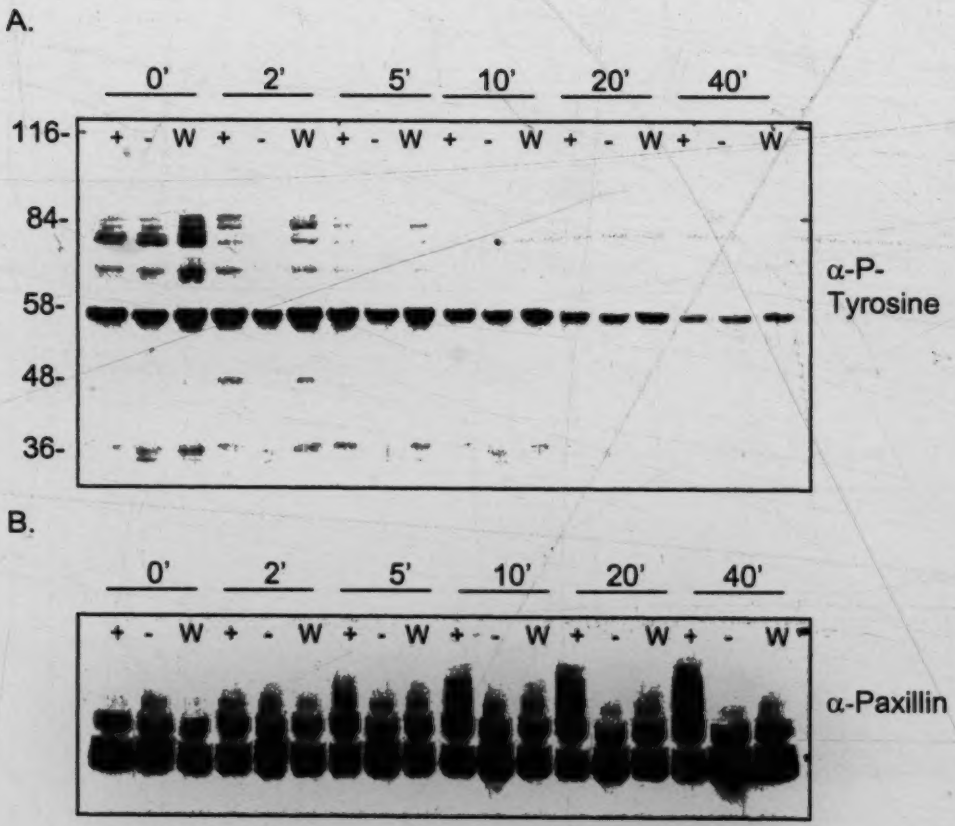


Figure 11. **Paxillin Phosphorylation, stimulated with Target cells is dependent on PI3K activity**. AB.1 CTL clones were pretreated with 50 nM wortmannin for 30 minutes at 37°C, or left untreated. CTL were then stimulated through coculture with L1210 or with L1210$^{kb/Dd}$ for the indicated time course. Post nuclear, reduced, whole cell lysates were blotted using antibody specific for phospho-tyrosine (A), and paxillin (B). ((+) AB.1 + L1210 $^{kb/Dd}$,(-) AB.1 + L1210, (W) AB.1 + L1210 $^{Kb/Dd}$ + 50 nM wortmannin). This experiment was repeated 3 times with similar results.

treatment. Given that paxillin is a cytoskeleton-associated protein, and that its phosphorylation is dependent on PI3K activity, our data implicate a unique role for PI3K activity in cytoskeletal rearrangement through regulation of paxillin.

3.7 PI3K plays a role in CTL spreading on immobilized anti-CD3 coated plates.

One of the most striking characteristics of CTL stimulated with immobilized anti-CD3 is the dramatic cytoskeleton driven, morphological change they undergo; after approximately 20 minutes, the CTL spread out and become flattened. It is our hypothesis that this spreading is comparable to the cytoskeletal rearrangements that occur upon CTL/target cell interaction. In support of this, CTL stimulated with immobilized anti-CD3 degranulate to the same extent, as cells stimulated with target cells whereas CTL stimulated with soluble or cross-linked anti-CD3 do not. Because degranulation and the phosphorylation of the cytoskeleton associated protein paxillin are dependent on PI3K activity, and because PI3K has been implicated in playing a role in cytoskeletal events in other cell types, we wanted to address if PI3K activity is required for the cytoskeletal events that result in CTL spreading. To answer this question, we stimulated cells with immobilized anti-CD3, and compared the morphology of wortmannin pre-treated to non-treated CTL. Figure 12A shows that untreated cells spread on an anti-CD3 coated plate after 20

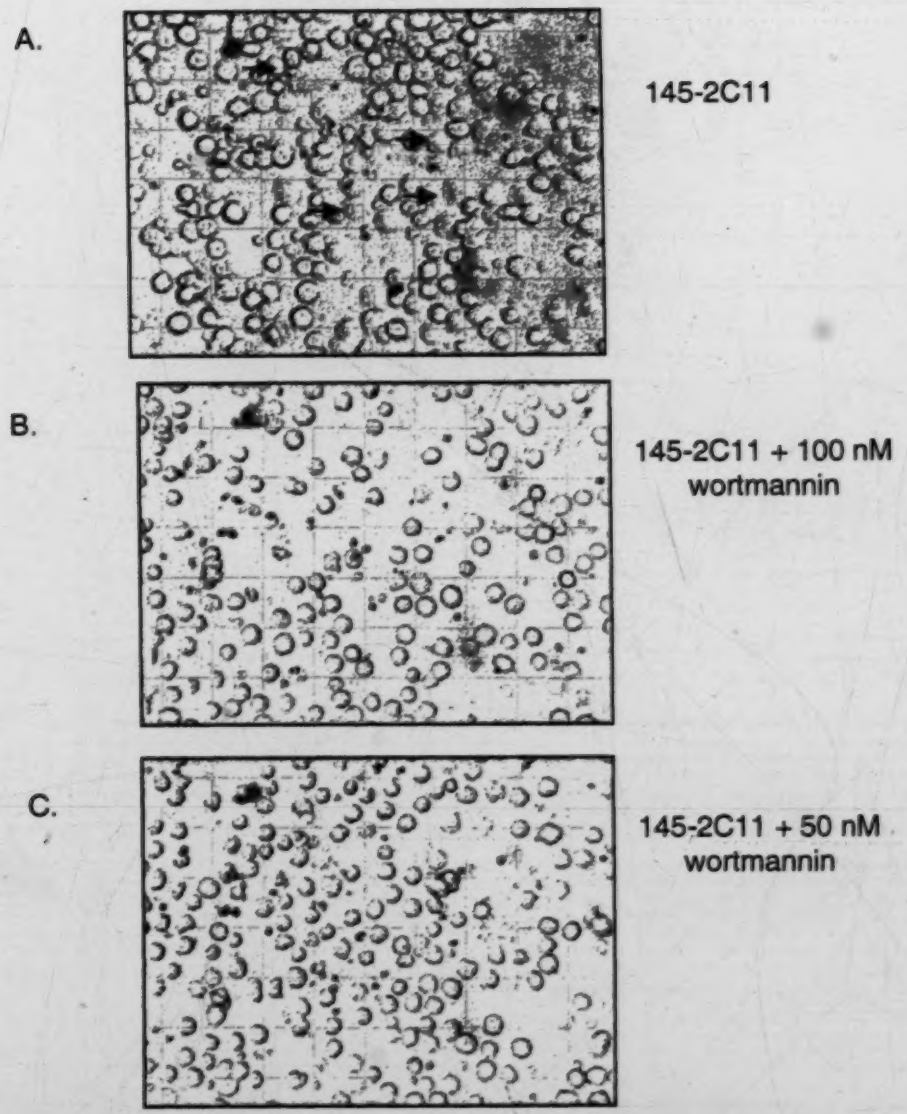


Figure 12. **PI3K plays a role in CTL spreading on immobilized α-CD3.** AB.1 CTL clones were pretreated with the indicated concentrations of wortmannin followed by stimulation with immobilized 145-2C11. At 25 minutes spreading was visualized using light microscopy. Arrows indicate cells that have spread. This experiment was repeated 3 times with similar results.

minutes. Compared to control cells, AB.1 pre-treated with wortmannin are inhibited from spreading on the antibody-coated surface (Figure 12B. and (figure 12C). Ly 294002 pre-treatment also inhibited spreading at concentrations of 50 and 10 μM (data not shown).

3.8 Wortmannin inhibits conjugate formation between CTL and target cells.

Given that cell/cell adhesion directly relies on cytoskeletal rearrangement and given that PI3K appears to play a role in CTL spreading on anti-CD3 coated plates, we hypothesized that PI3K may play a role in the formation of, or stabilization of CTL/target cell conjugates, the formation of which is necessary for effector function. To test this hypothesis we compared the percent of conjugates formed in untreated samples containing CTL (labeled with green lipophilic dye) with targets (labeled with red lipophilic dye) to samples where the CTL had been pre-treated with wortmannin and then cocultured with targets. At various time points, conjugates were fixed with 4% paraformaldyhyde and counted using Flow Cytometric analysis. Figure 13 shows that wortmannin treated CTL form fewer conjugates with targets than untreated CTL. The figure also shows that conjugate formation is blocked when the interaction between LFA-1 and ICAM-1 is interrupted using anti-LFA-1 antibodies. This data suggests that PI3K plays a role in TCR triggered LFA-1 mediated adhesion.

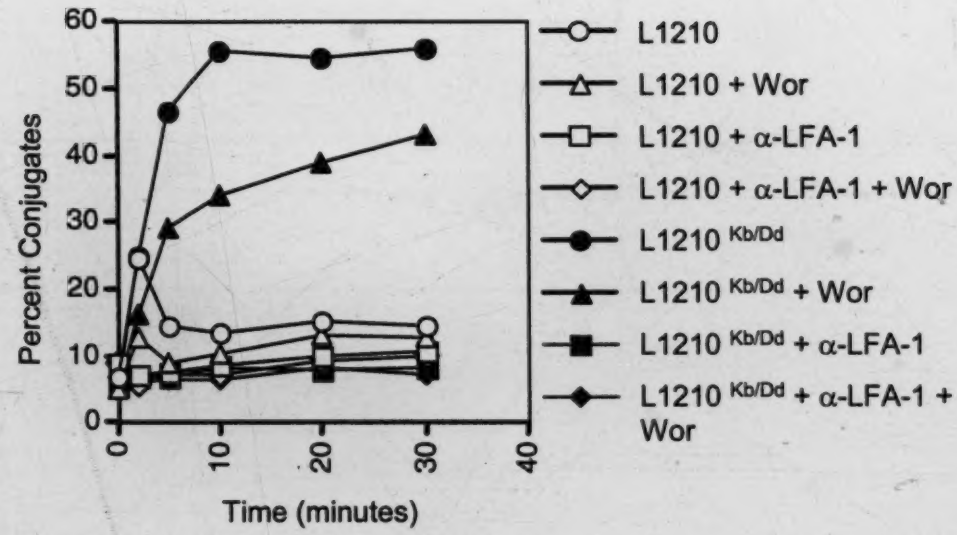
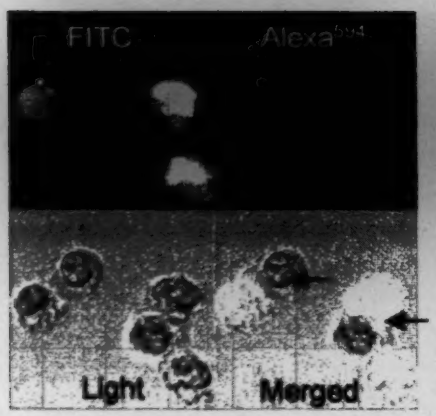


Figure 13. **PI3K activity is required for the optimal formation of CTL /Target cell conjugates**. AB.1 CTL clones were stained with green lipophilic dye followed by pretreatment with 50 nM wortmannin, or left untreated. L1210 and L1210 $^{Kb/Dd}$ were stained with red lipophilic dye. Targets and CTL were co-cultured at 37°C and conjugates were allowed to form. At the indicated time point's conjugates were vortexed, fixed and quantified using Flow Cytometric analysis. This experiment is representative of 5 experiments all showing a similar pattern of inhibition.

3.9 MTOC polarization requires conjugate formation in a PI3K dependent manner.

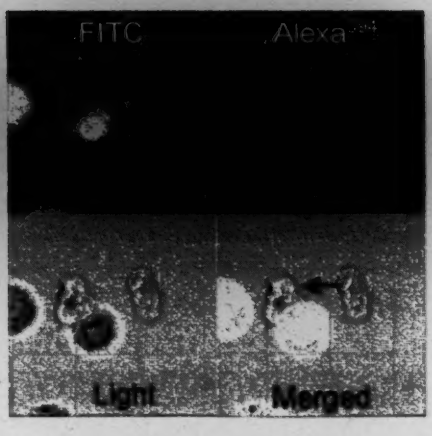
Others have shown in NK cells that PI3K activity is required for MTOC (microtubule organizing center) polarization (163). Given our data that PI3K plays a role in multiple activities required for CTL degranulation (cell spreading, conjugate formation, paxillin phosphorylation), combined with the fact that MTOC polarization is a cytoskeletal event also required for degranulation, and found to be PI3K dependent in other cell types, we addressed whether PI3K activity is required for MTOC polarization in CTL. MTOC reorientation is an early event in CTL activation that occurs shortly after conjugate formation, wherein the MTOC becomes polarized towards the target cell. This reorientation involves the repositioning of the Golgi apparatus and facilitates directed secretion of cytolytic granules. Interestingly, paxillin has been shown to associate with γ tubulin, which is the major tubulin component of the MTOC. In order to address whether PI3K plays a role in MTOC polarization in CTL, we counter stained target cells using FITC, drug treated CTL with 50 nM wortmannin, or left them untreated, and then cocultured unstained CTL with green targets at 37°C for 20 minutes. We then applied drops of cocultured cells onto poly-l-lysine coated cover slips, fixed, permeablized and stained the cells with red pericentrin (antibody specific for MTOC). Using confocal microscopy, we visualized MTOC polarization in individual conjugates and quantified polarization in multiple conjugates. Figure 14A shows a positive control where the MTOC

A.



AB.1+ L1210 $^{kb/Dd}$
(+ 've control)

B.



AB.1 + L1210 $^{Kb/Dd}$ + 50 nM wortmannin

C.

	Negative control L1210 + AB.1	Positive control L1210 $^{Kb/Dd}$ + AB.1	Experimental L1210 $^{Kb/Dd}$ + AB.1 + 50 nM Wor
Total AB.1	100	100	100
Conjugates	12	65	32
Polarized MTOC	5	54	23
% polarization	NA	84%	72%

Figure 14. **PI3K dependent conjugate formation is required for MTOC polarization.** AB.1 CTL clones were pretreated with 50 nM wortmannin for 30 minutes at 37°C followed by coculture with targets prestained with FITC. After 20 minutes conjugates were fixed and solublized with cytofix/cytoperm and the MTOC stained with pericentrin primary antibody and red labeled anti-rabbit secondary antibody. MTOC polarization was visualized using confocal microscopy. (Arrows indicate polarized MTOC). This experiment was repeated 3 times with similar results. (Note: we do not typically observe decreased staining in wortmannin treated cells.)

of the untreated CTL is polarized towards the target L1210 $^{Kb/Dd}$. Figure 14B shows that pre-treatment with wortmannin can inhibit the polarization of the MTOC. In the negative controls, the numbers of conjugates were very low, and MTOC polarization, where it occurred, could not be attributed to known mechanisms of conjugation. Figure 14C reveals that although the number of conjugated CTL with polarized MTOC in drug treated samples is only around 13% less than in non-treated, drug treated CTL form approximately 50% fewer conjugates than non-treated. This suggests that although wortmannin does inhibit conjugate formation, it does not appear to affect MTOC reorientation.

3.10 Summary of Results

The results detailed in this chapter consist of three major findings. First, PI3K activity is required for initiating CTL degranulation, but is not required for the process of degranulation since PI3K inhibitors do not inhibit TPA and Ionomycin induced degranulation. Second, paxillin is dependent on PI3K activity for complete phosphorylation in CTL. Third, PI3K plays a role in cytoskeletal rearrangement and adhesion. Together this chapter supports the hypothesis that PI3K contributes to cytoskeletal rearrangement and is required for CTL effector function.

Chapter IV

PI3K activity is required for the phosphorylation of PKC θ

4.1 Introduction

Our previous results indicate that PI3K activity is required for the phosphorylation of specific signaling proteins that become activated early upon TCR stimulation in CTL. The first protein whose phosphorylation we found to be dependent on PI3K activity, the cytoskeletal-associated protein paxillin, plays an important role in cytoskeletal rearrangement. Given the involvement of PI3K in the regulation of paxillin, together with its role in CTL spreading, we hypothesized that PI3K may play a role in the regulation of and correspondingly the phosphorylation of other cytoskeleton associated proteins. Our first approach to explore this possibility was to look more closely at previous findings that PI3K activity is required for the phosphorylation of the guanine nucleotide exchange factor Vav-1.

4.2 PI3K activity is not required for Vav phosphorylation in CTL.

Given that PI3K plays a role in the phosphorylation of paxillin and given that others have shown that the PH domain of Vav-1 (a Rho-family GEF implicated in cytoskeletal rearrangements) contributes to its phosphorylation and activation (140), we next addressed if PI3K plays a role in the phosphorylation of Vav-1. In order to address this question, we immunoprecipitated Vav-1 from CTL, pre-treated with PI3K inhibitors, and

stimulated with immobilized anti-CD3 for 20 minutes (a time of peak Vav-1 phosphorylation in our AB.1 CTL clones). We then looked specifically at the tyrosine phosphorylation of Vav by probing the membrane with antibody specific for phospho-tyrosine containing proteins. Figure 15 (top panel) shows that the phosphorylation of Vav is not affected by wortmannin treatment. To ensure that the protein pulled down by immunoprecipitation was indeed Vav; we re-probed the blots with antibodies specific for Vav (middle panel) and found that Vav was successfully recovered from the CTL lysates.

Importantly, other studies have shown that Vav and PKC θ can associate physically with one another. Moreover, PKC θ is a unique isoform of PKC, highly expressed in T cells, and implicated as a potential bridge between cytoskeletal rearrangement and downstream signalling events (164). Given the relevance of PKC θ to Vav and potentially to the cytoskeleton, we stripped and re-probed the blots with antibody specific for PKC θ to see if the interaction between PKC and Vav was affected by wortmannin treatment. In our CTL we found no detectable association between Vav and PKC, (bottom right panel) however, in the cell lysates we noticed that inhibiting PI3K activity resulted in inhibiting the conversion of PKC from a faster to a slower migrating species. Instead of having an effect on Vav, we were intrigued to find that wortmannin treatment affects the mobility of PKC θ, seemingly independent of Vav. Figure 15 represents one of three experiments, all with similar results.

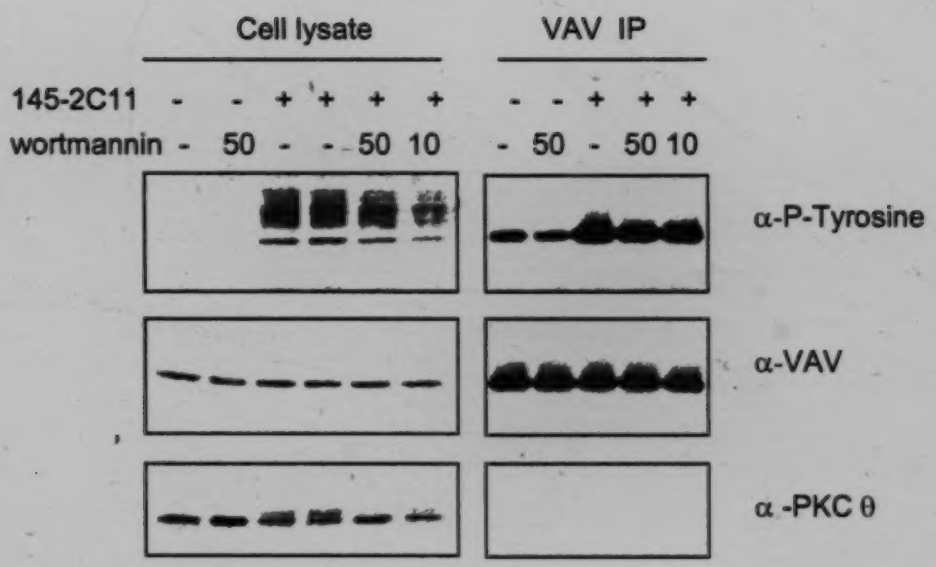


Figure 15. **PI3K activity is not required for Vav phosphorylation in CTL, but may effect the mobility of PKC θ.** AB.1 CTL clones were pretreated with 50 nM and 10 nM wortmannin for 30 minutes at 37°C followed by stimulation with immobilized 145-2C11. Vav immunoprecipitates and reduced whole cell lysates were blotted with phospho-tyrosine (top panel), Vav (middle panel) and PKC θ antibodies.

4.3 PI3K activity is required for the formation of a higher molecular weight form of PKC θ, and for the phosphorylation of ERK.

In order to further study the shift in PKC θ with regard to AB.1 stimulation, and the inhibition of the shift with wortmannin treatment, we performed a similar drug titration assay to what is seen in figure 8. Figure 16 A and B (middle panels) show that wortmannin or Ly 294002 treatment of CTL prior to stimulation with immobilized anti-CD3 inhibits the shift in PKC θ from a lower to a higher MW form in a concentration dependent manner. Also, because MAP kinase activation has in some systems been linked to PKC signaling, and because PI3K has been suggested in other experimental systems to be upstream of ERK activation, we also probed the blots with antibodies specific for phospho-ERK and found as shown in figure 16A and B (bottom panels) that the phosphorylation of ERK depends on PI3K activity (as illustrated through inhibition of the shift of ERK from a faster migrating, unphosphorylated form to a slower migrating phosphorylated form).

4.4 PI3K activity is required for the phosphorylation of PKC θ.

To determine if the shift in PKC θ from a faster to a slower migrating species was due to phosphorylation, we immunoprecipitated PKC θ from CTL pretreated with wortmannin and stimulated with immobilized anti-CD3 for 20 minutes (a time point of optimal PKC-phosphorylation). In addition, we treated one sample of immunoprecipitated PKC θ with alkaline phosphatase to remove phosphate groups from the protein. Figure 17 (top panel) shows

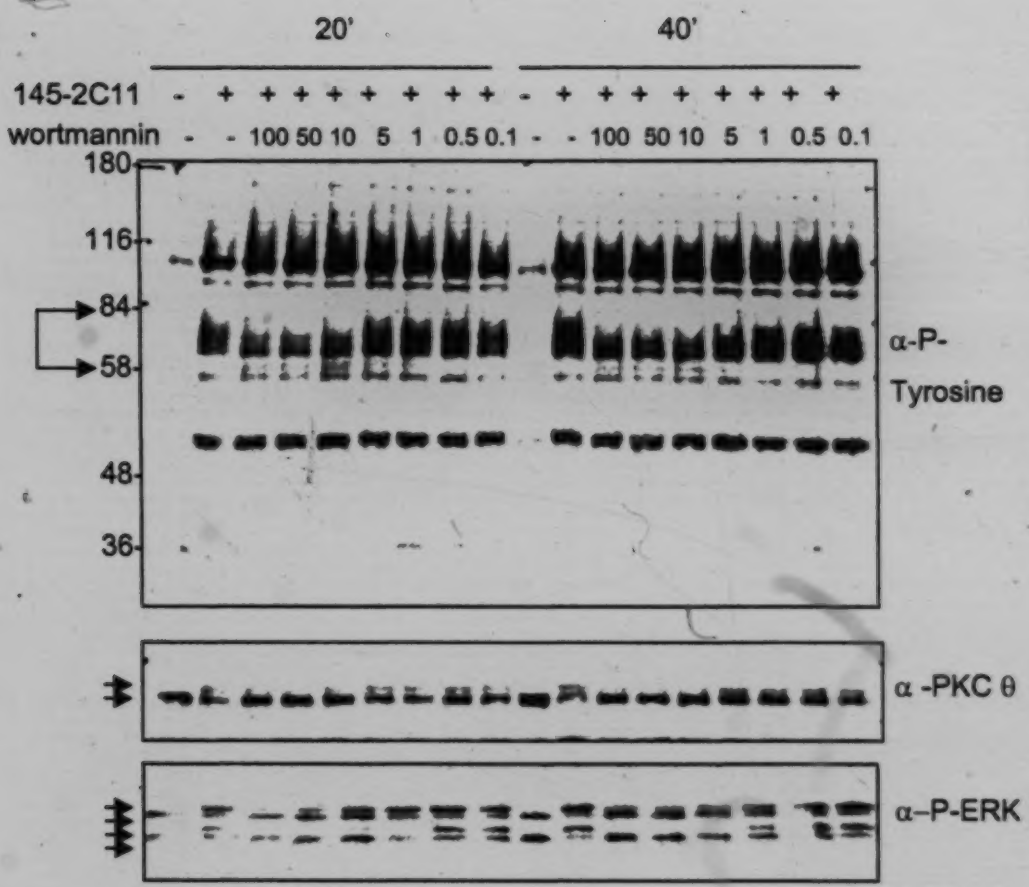


Figure 16. A. **PI3K activity is required for the formation of a slower migrating species of PKC θ and for the phosphorylation of ERK.** AB.1 CTL clones were pretreated with 50 nM wortmannin for 30 minutes at 37°C followed by stimulation with immobilized 145-2C11. Reduced whole cell lysates were blotted with phospho-tyrosine (top panel), PKC θ (middle panel) and phospho-ERK (bottom panel) antibodies. This experiment was repeated 3 times with similar results.

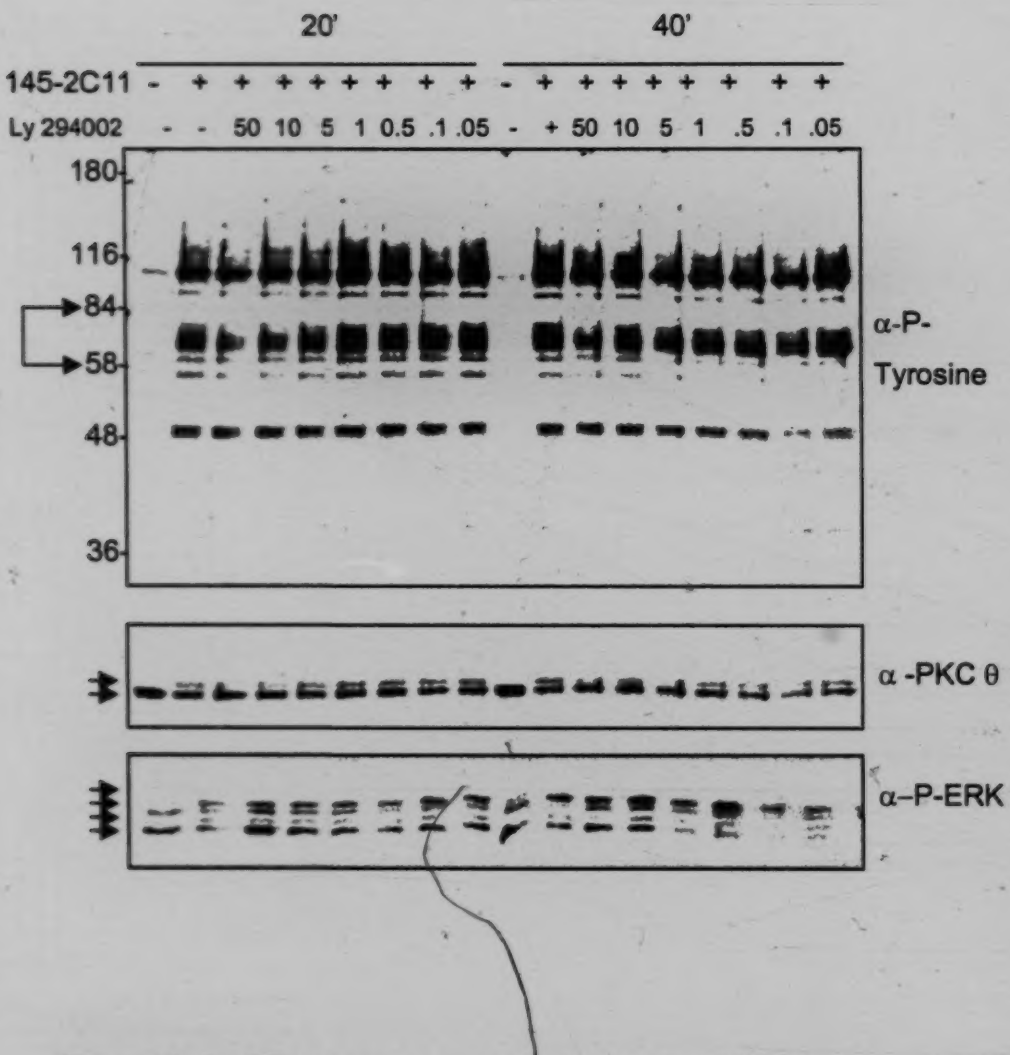


Figure 16. B. CTL clones were pretreated with Ly 294002 and treated as in A.

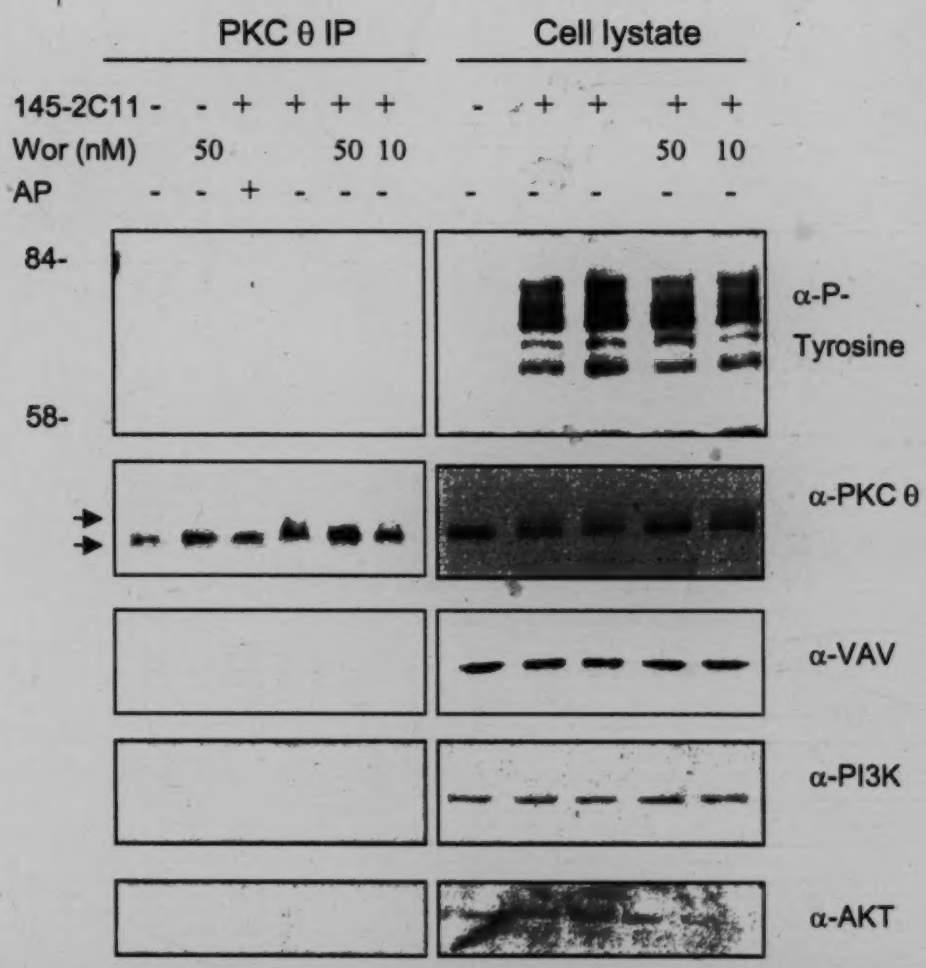


Figure 17. **PKC θ is dependent on PI3K activity for phosphorylation but does not co-immunoprecipitate with PI3K nor with AKT.** AB.1 CTL clones were pretreated with 50 nM and 10 nM wortmannin for 30 minutes at 37°C followed by stimulation with immobilized 145-2C11. Reduced whole cell lysates and IPs were blotted with phospho-tyrosine (1st panel), PKC θ (2nd panel), Vav (3rd panel), PI3K (4th panel), and AKT antibodies (5th panel). AP designates Alkaline phosphatase treatment. This experiment was repeated 3 times with similar results.

that PKC θ is not tyrosine phosphorylated to a detectable extent, although repeats of this experiment suggest a small degree of tyrosine phosphorylation. Figure 17 also shows that the shift in PKC θ is due to phosphorylation as alkaline phosphatase treatment inhibits the formation of the upper band (2nd panel). The second panel also shows that wortmannin treatment inhibits the serine/threonine phosphorylation of PKC θ. As expected, we were unable to detect a physical association between PKC and VAV (3rd panel), nor could we detect an association between PKC θ and PI3K (4th panel), nor between PKC θ and AKT (5th panel).

As with paxillin, we wanted to address the kinetics of PKC θ phosphorylation, when it occurs and how long is it maintained during CTL activation. As well we wanted to address whether inhibiting PI3K had an effect on the initial formation of the phosphorylated form, or in maintaining the phosphorylated form. Figure 18 shows the results of a kinetic assay addressing these questions wherein CTL were either pre-treated with 50 nM wortmannin or left untreated, and stimulated with immobilized anti-CD3 for the indicated times before being lysed under reducing conditions. Figure 18A represents a control where whole cell lysates were blotted with antibodies specific for tyrosine-phosphorylated protein and shows that tyrosine phosphorylation increases over time. Figure 18B shows that PKC θ becomes phosphorylated after approximately 10 minutes of stimulation with anti-CD3. The phosphorylated form remains present throughout a 60-minute time course of stimulation. Wortmannin treatment inhibits the phosphorylation of

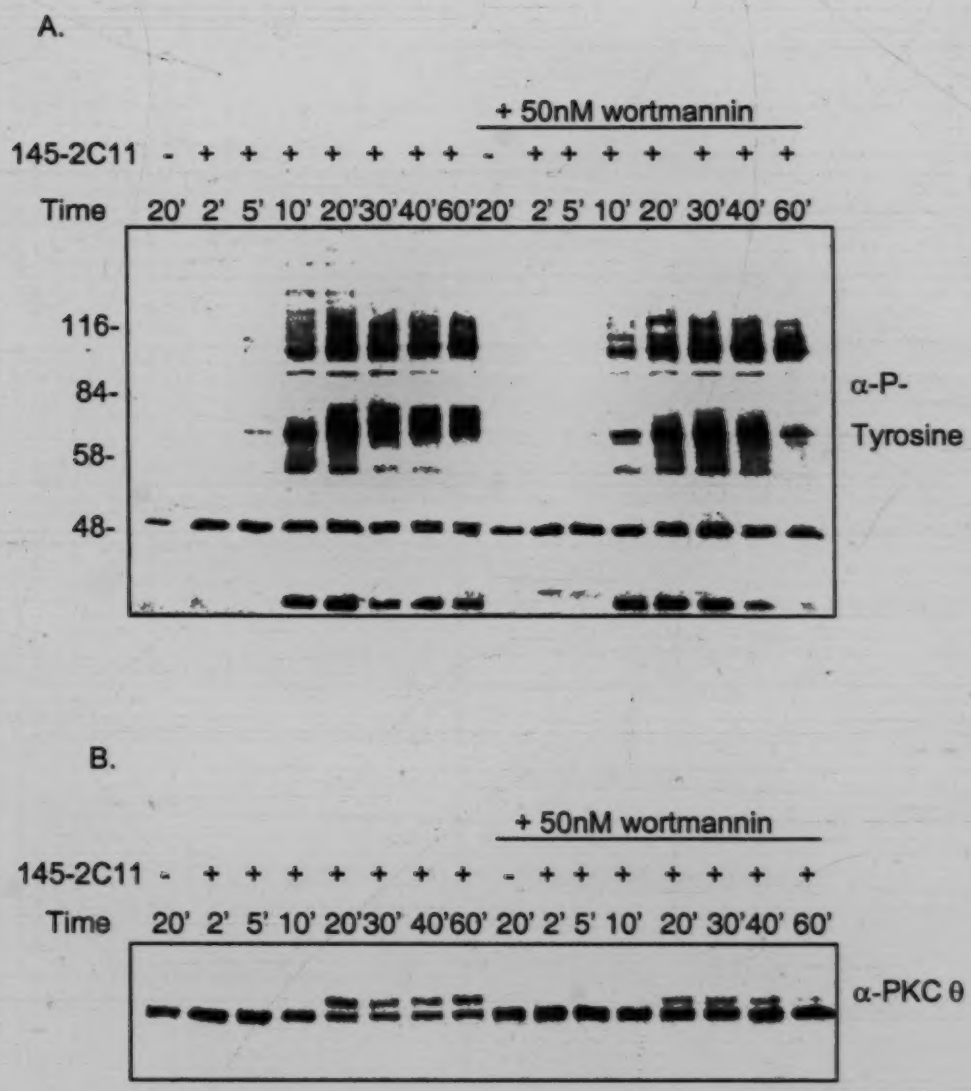


Figure 18. **PKC θ phosphorylation, stimulated by immobilized α-CD3, requires PI3K activity**. AB.1 CTL clones, pretreated with 50 nM wortmannin, or left untreated, were stimulated with 145-2C11 over a time course. Reduced whole cell lysates were blotted using antibodies specific for phospho-tyrosine (A) and PKC θ (B). This experiment was repeated 3 times with similar results.

PKC θ, and this inhibition is more severe at later versus earlier time points. An alternative explanation is that wortmannin could be stabilizing the lower band by increasing the expression of, or more likely inhibiting the degradation of the faster migrating species. To extend our observation to CTL stimulated with target cells, we first wanted to assess the expression pattern of PKC θ in our CTL versus the target cells. All cells, analyzed for PKC θ expression have been shown to express the protein under both basal and stimulatory conditions. Figure 19A shows that AB.1 CTL clones express PKC θ whereas our L1210 lymphoma target cell line does not. Figure 19B shows that coculture with target cells stimulates protein tyrosine-phosphorylation. Figure 19B also shows that when CTL are stimulated through coculture with target cells following pre-treatment with wortmannin the phosphorylation of PKC θ is inhibited, as compared with untreated controls. Moreover, the inhibition is more severe at later time points (20 and 40 minutes), similar to what is seen in CTL stimulated with immobilized anti-CD3.

4.5 TPA stimulates the phosphorylation of PKC θ independently of PI3K activity.

TPA, being a diacyl glycerol analogue, stimulates the phosphorylation of classical PKCs. Given that TPA and ionomycin stimulate degranulation of CTL independently of PI3K activity, we hypothesized that TPA would stimulate the phosphorylation of PKC θ independently of PI3K activity. To show this we first needed to determine if TPA stimulated the phosphorylation

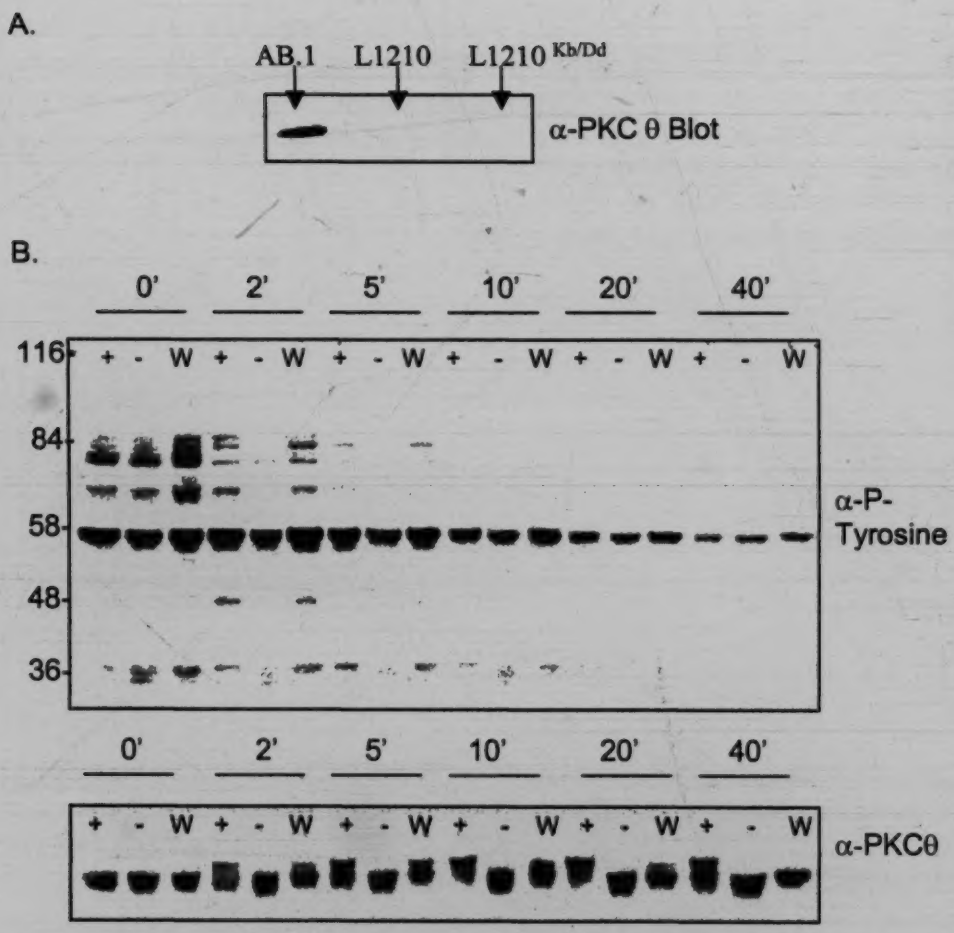


Figure 19. **PKC θ phosphorylation, stimulated with Target cells is dependent on PI3K activity**. (A) reduced, whole cell lysates of AB.1 CTL clones and L1210 lymphomas were blotted for PKC θ. (B) AB.1 CTL clones were pretreated with 50 nM wortmannin for 30 minutes at 37°C, or left untreated. CTL were then stimulated through coculture with L1210 or with L1210$^{kb/Dd}$ for the indicated time course. Post nuclear, reduced, whole cell lysates were blotted using antibody specific for phospho-tyrosine (top panel), and PKC θ (bottom panel). ((+) AB.1 + L1210 $^{kb/Dd}$,(-) AB.1 + L1210, (W) AB.1 + L1210 $^{Kb/Dd}$ + 50 nM wortmannin). This experiment was repeated 3 times with similar results.

of PKC θ. Thus we immunoprecipitated PKC θ from CTL stimulated with TPA and treated one sample with alkaline phosphatase. Figure 20A shows that the shift in PKC θ stimulated by TPA is a result of phosphorylation as alkaline phosphatase reduces the shift to the basal level. Figure 20B (middle panel) shows that PKC θ remains phosphorylated in whole cell lysates from CTL pretreated with wortmannin and stimulated with TPA as compared with PKC from cell lysates stimulated with immobilized anti-CD3. As expected, stimulation with immobilized anti-CD3 results in the phosphorylation of AKT downstream of PI3K (20B bottom panel) however; we cannot detect AKT phosphorylation in CTL stimulated with TPA. Together these results suggest that PI3K contributes to the phosphorylation of PKC θ via an alternative pathway from that which is stimulated by TPA alone.

4.6 PKC θ exists in an unphosphorylated form in the CTL membrane under basal conditions.

Given that serine/threonine phosphorylation of PKC θ is PI3K dependent, we wanted to address the effect of phosphorylation on the localization of PKC θ. In order to answer this question, we first needed to assess the localization of PKC θ under basal and stimulatory conditions. We used the lack of expression of PKC θ in target cells to specifically address the localization of PKC θ in CTL upon target cell stimulation. CTL were stimulated by coculture with target cells for 5 and 20 minutes. Cell lysates were fractioned into crude, soluble, membrane and cytoskeletal components

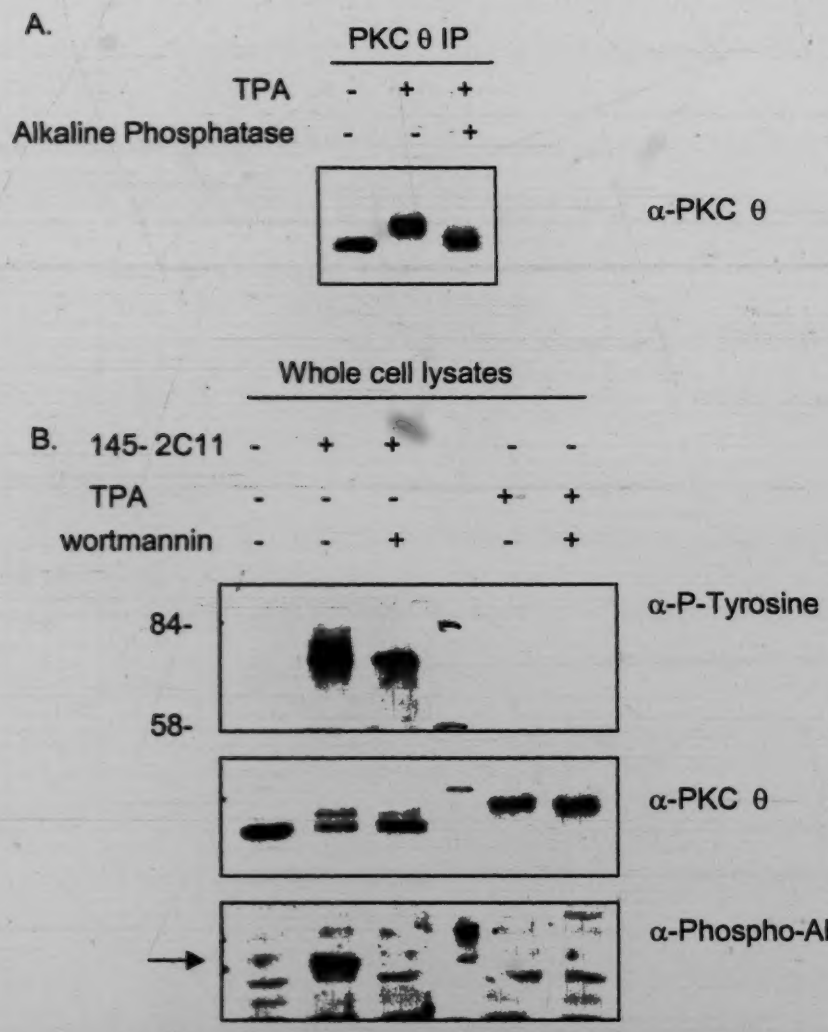


Figure 20. **TPA stimulates the phosphorylation of PKC θ via a pathway independent of PI3K.** (A) PKC θ was Immunoprecipitated from AB.1 left unstimulated, or stimulated with TPA. One of two stimulated samples was treated with alkaline phosphatase. IPs were blotted with antibody specific for PKC θ. (B) AB.1 CTL clones were pretreated with 50 nM wortmannin for 30 minutes at 37°C followed by stimulation with immobilized 145-2C11 or with TPA. Reduced whole cell lysates were blotted with phospho-tyrosine (top panel), PKC θ (middle panel), and phospho-AKT (bottom panel) antibodies. These experiments were repeated 3 times with similar results.

via ultra-centrifugation methods and Triton X 100 solublization. These fractions were then analyzed through western blotting to assess the localization of PKC θ. Figure 21 (top panel) shows that in the crude fraction, stimulation with antigen-bearing target cells results in tyrosine phosphorylation of proteins. Figure 21 (2nd panel) shows that PKC θ is present at the membrane under basal conditions, and is phosphorylated under stimulatory conditions. Figure 21 (3rd panel) shows that CD45 is present in the crude fraction, as well as the membrane fraction, but not in the soluble fraction illustrating that separation between the fractions is complete. Figure 21 (4th panel) illustrates that the majority of paxillin in both the CTL and target cells is present in the insoluble fraction, and that paxillin is phosphorylated upon target cell stimulation.

4.7 PI3K plays a role in the phosphorylation of PKC θ at the membrane.

In other systems PKC θ relocalizes to the membrane upon cell stimulation where it participates in forming the c-SMAC (104). Given that the phosphorylation of PKC θ in our CTL is dependent on PI3K, and given that wortmannin treatment inhibits that phosphorylation of PKC θ, we wanted to use this experimental system to address the effect of PI3K-dependent phosphorylation on PKC with regard to localization. So, we used the method outlined for figure 21 to illustrate the effect of wortmannin treatment on PKC θ localization. Our results showed that PKC θ association with the membrane does not change significantly with wortmannin treatment; however, as

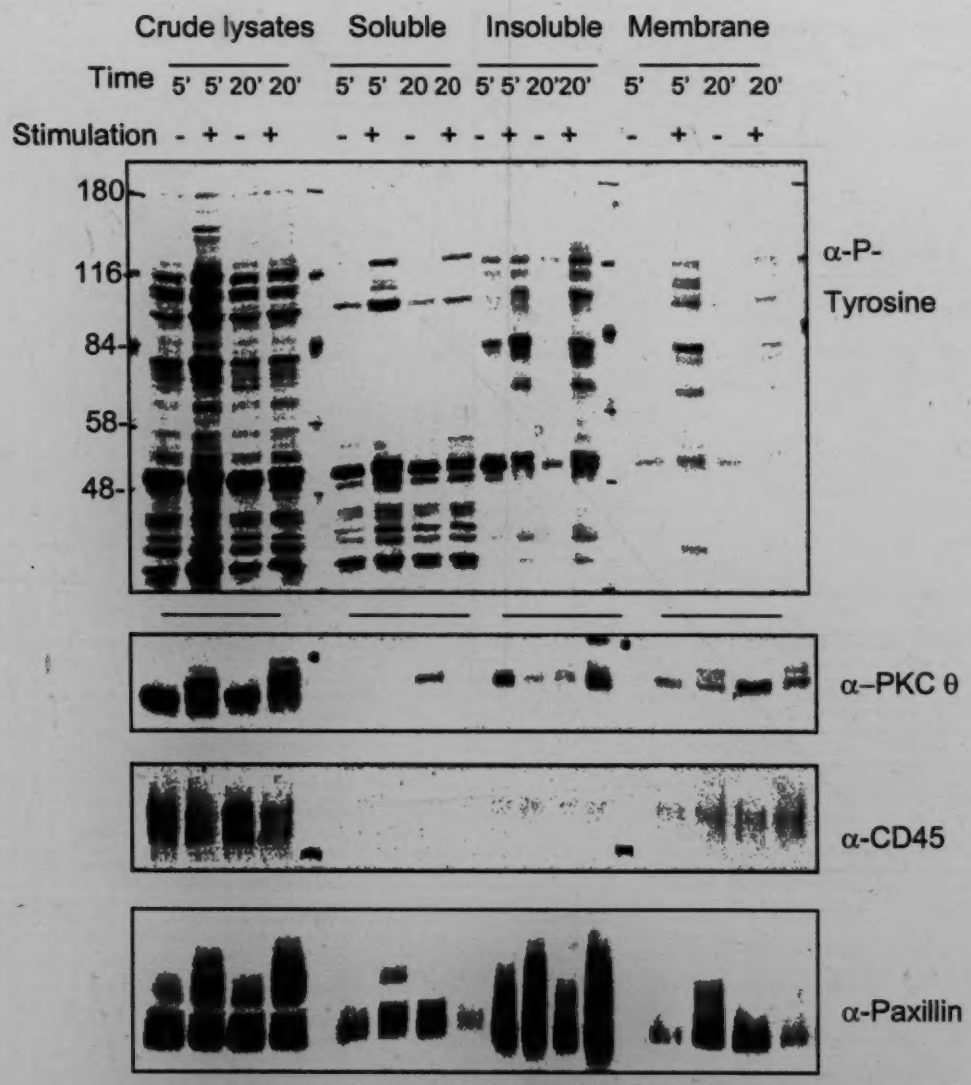


Figure 21. **PKC θ is localized at the membrane regardless of stimulation.** AB.1 CTL clones were stimulated by coculture with target cells for the indicated time periods. Conjugates were lysed and the cell components separated into the indicated fractions. Reduced proteins in each fraction were blotted with phospho-tyrosine (top panel), PKC θ (2nd panel) CD45 (3rd panel) and paxillin antibodies (4th panel). ((+) indicates CTL cocultured with antigen bearing targets, (-) indicates CTL cocultured with control targets).

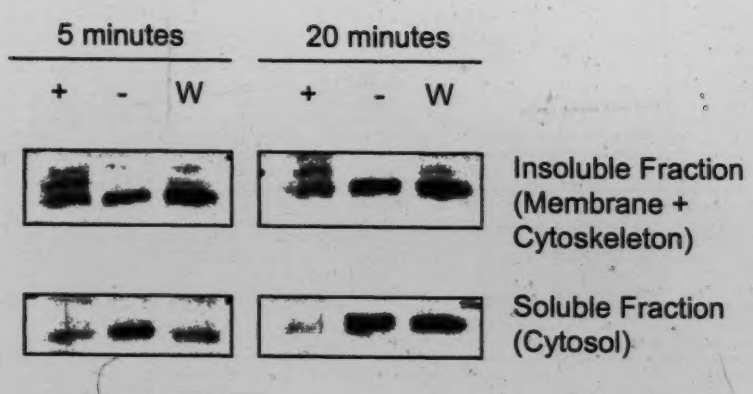


Figure 22. **PKC θ localization with Drug Treatment** AB.1 CTL clones, where indicated, were pretreated with wortmannin, and stimulated by coculture with target cells for the indicated time periods. Conjugates were lysed and post nuclear cell lysates, separated into the indicated fractions. Reduced proteins in each fraction were blotted with antibody for PKC θ. ((+) AB.1 + L1210 $^{kb/Dd}$, (-) AB.1 + L1210, (W) AB.1 + L1210 $^{kb/Dd}$ + 50 nM wortmannin.) This experiments was repeated 5 times with similar results.

expected, the phosphorylation of PKC θ at the membrane is inhibited with wortmannin treatment (figure 22 top panel).

4.8 The phosphorylation of PKC θ is dependent on the integrity of the cytoskeleton.

The presence of PKC θ in lipid rafts, as well as PKCs' dependency on PI3K activity for phosphorylation, suggest that PKC θ phosphorylation is downstream of cytoskeletal rearrangement. We wanted to determine if PKC phosphorylation actually requires cytoskeletal rearrangement to take place, thus we performed an assay whereby cytoskeletal integrity was disrupted using cytochalasin E after a 15-minute stimulation with immobilized anti-CD3. Figure 23 (2nd panel) shows that the phosphorylation of PKC θ is inhibited when the integrity of the cytoskeleton is disrupted. Moreover, the degree of inhibition of phosphorylation mirrors that seen with wortmannin treatment. This suggests that PI3K contributes to cytoskeletal rearrangement and thus in the phosphorylation of PKC θ.

4.9 Summary of Results

The results detailed in this chapter consist of two major findings. First, PI3K activity is required for the phosphorylation of PKC θ and ERK. Second, PKC θ is downstream of and dependent on cytoskeletal integrity for its phosphorylation. Together this data suggests that PI3K plays a key role in cytoskeleton-dependent phosphorylation of proteins involved in key signalling processes it CTL.

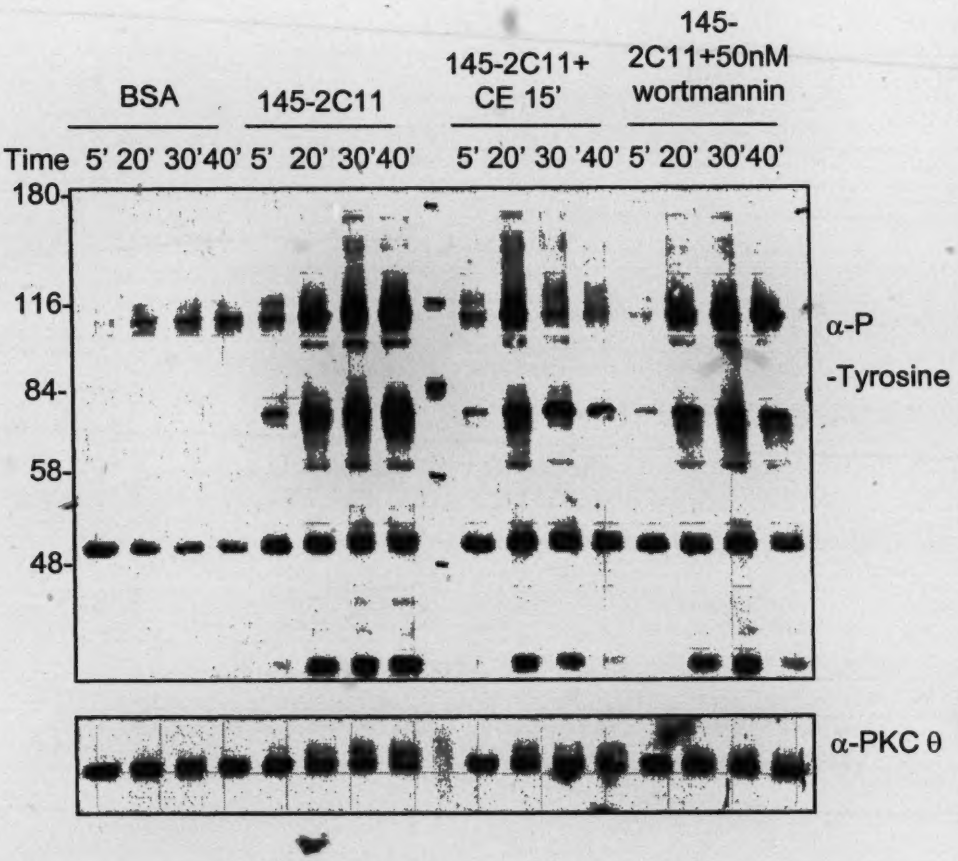


Figure 23. **Phosphorylation of PKC θ depends on an intact cytoskeleton**. AB.1 CTL clones were pretreated with wortmannin, or left untreated for 30 minutes prior to stimulation with immobilized 145-2C11. At 15 minutes cytochalasin E was added to the indicated samples. Reduced whole cell lysates were blotted using phospho-tyrosine (top panel) and PKC θ antibodies (bottom panel). This experiment was repeated 3 times with similar results.

Chapter V

Discussion

5.1 Summary of Results

This study reveals that PI3K activity is required for CTL activation and degranulation. The findings in this study support previous work also suggesting that PI3K is required for CTL degranulation, but not for Fas Ligand mediated killing (161). Although Fuller *et al.* showed in 1999 that PI3K is required for degranulation; their research did not delineate a clear role for PI3K in degranulation. Our data support a role for PI3K in upstream signaling events that facilitate degranulation. We show that PI3K activity is required for degranulation as stimulated through the TCR, but not for degranulation stimulated by phorbol ester and calcium ionophore. Moreover, in contrast to results in NK cells showing that PI3K is involved in MTOC polarization and degranulation, our results suggest that PI3K plays a role upstream of MTOC polarization in a process that precedes polarization such as CTL/target cell conjugation or adhesion. Our finding that PI3K plays a role upstream in CTL signaling, rather then downstream in the process of degranulation, is novel and opens up the field for research into the mechanism of PI3K involvement. This study highlights some of the potential mechanisms for PI3K involvement and provides evidence that PI3K plays a critical role in the signaling events that trigger cytoskeletal rearrangement.

5.2 Upstream Mechanisms of PI3K activity in CTL activation and effector function

The potential mechanisms of PI3K involvement in CTL activation and effector function include: a role in the localization and phosphorylation of proteins involved in cytoskeletal rearrangement, cell spreading, and adhesion, as well as a role in the localization and phosphorylation of potential adaptor proteins that link cytoskeletal rearrangement and downstream signaling events.

5.2.1 Cytoskeleton Associated Proteins Regulated by PI3K

PI3K has been implicated in the activity of the Rho-family G-proteins Rac-1, Rho and Cdc42, which contribute to the dynamic organization of the actin cytoskeleton and the clustering of integrins (127) (138) (139). PI3K is thought to regulate these G-proteins by contributing to the activation of upstream guanine nucleotide exchange factors (GEFs) that facilitate G-protein activation. All GEFs are members of the dbl family of proteins and as such contain PH domains (165). One example of a GEF that has been well studied in the context of T cells is Vav-1. In a number of experimental systems, Vav-1 has been shown to be a key contributor to T cell activation and cytoskeletal rearrangement. Some of the findings include: 1) that the proper function of Vav is important for receptor induced activation of MAP kinase ERK, and the transcription factors NFAT and NFκB (46), (47), (166), 2) Vav deficient T cells fail to undergo TCR receptor clustering and actin polymerization (94) (47) (96), and 3) Vav signaling is required for lipid raft

clustering in T cells; lipid rafts do not translocate to the immunological synapse in Vav deficient T cells upon antigen stimulation (167). Moreover, the PH domain of Vav-1 has been shown to associate with PIP3, an interaction that promotes tyrosine kinase mediated activation of this enzyme (140). In our AB.1 CTL clones, Vav-1 is phosphorylated upon stimulation with immobilized anti-CD3 antibodies, however in contrast with previous work (168) Vav-1 in AB.1 remains phosphorylated when PI3K is biochemically inhibited. Thus, a role for PI3K in the activation of Vav is unclear in our system.

As well, PIP3 generated by PI3K, has been shown to contribute to the activation of WASp; PIP3 interacts with the PH domain of WASp and facilitates its localization to the membrane (73) (141) where it is activated and takes on an open conformation. Open conformation WASp binds Arp2/3, triggering Arp2/3 association with actin resulting in actin polymerization. PI3K may play a role in cytoskeletal rearrangement by contributing to the localization, phosphorylation and activation of cytoskeleton-associated proteins including Vav, and WASp, thus facilitating CTL effector function.

5.3 PI3K Regulates the Phosphorylation of Paxillin

Our results clearly show that PI3K activity is required for the phosphorylation of paxillin; paxillin is phosphorylated upon CTL stimulation with immobilized anti-CD3 antibody, or with target cells, and phosphorylation is inhibited when PI3K activity is inhibited. Paxillin is an important

cytoskeletal protein that localizes to sites of adhesion with the extracellular matrix and becomes phosphorylated upon CTL activation (107) (108). The tyrosine phosphorylation of paxillin is thought to be important for cell spreading on ECM (109), and for adhesion dependent functions in leukocytes (110), although the signaling pathway that paxillin contributes to remains unclear. Paxillin is known to function as an adaptor molecule, providing docking sites for both structural (tensin, α-actin and vinculin) and regulatory proteins (focal adhesion kinase (FAK)) (106) and as such, is thought to play a key role in cytoskeletal rearrangement. Interestingly, phosphorylated paxillin has also been shown to associate with Lck (61). Taken together with our data, showing that paxillin is phosphorylated in a PI3K dependent manner, we suggest that paxillin may work downstream of PI3K to connect membrane proximal signaling events with cytoskeletal rearrangements involved in cell spreading and cell/cell adhesion.

5.3.1 Potential mechanism of PI3K-dependent Paxillin regulation

The finding that PI3K is required for paxillin phosphorylation is novel, thus, little has been done to assess the mechanism for PI3K involvement in paxillin phosphorylation. We propose two potential mechanisms. First, Paxillin directly associates with FAK (106), as does PI3K (160). It is possible that this physical interaction results in the phosphorylation of paxillin, either via conformational change in paxillin or via the ill-described protein kinase activity of PI3K. However, in our CTL clones, we cannot detect a physical

association between paxillin and PI3K, suggesting that another mechanism orchestrates the phosphorylation of paxillin in a PI3K dependent manner.

Paxillin has recently been found to exist in a complex of proteins that include PAK/Paxillin/p95PKL/and Pix β. Pix β, a novel Rac-1 guanine nucleotide exchange factor first identified in 1998, contains a PH domain (142) (143) and has been found in Rat Brain and Hela cells to be dependent on PI3K (144) for localization and activation. Assuming the same complex exists in CTL, it is plausible that the PH domain of Pix β plays a role in localization of the PAK/Paxillin/p95PKL/Pix complex to the membrane upon T cell activation. However, recent studies in Jurkat have suggested that in fact paxillin is absent from the complex (112). Although we have not been able to detect p95PKL, nor Pix β in our paxillin immunoprecipitations, we cannot rule out the possibility that this complex exists in our AB.1 CTL clones. The association of paxillin with p95PKL would suggest that PI3K regulates the phosphorylation of paxillin by contributing to the production of PIP3, which correspondingly binds the PH domain of Pix β (or Pix α) and facilitates the relocation of the PAK/Paxillin/p95PKL/Pix complex to the membrane where paxillin can become phosphorylated. In support of this model, Pak has been implicated in MAP kinase signalling (111); our data support a role for PI3K in the phosphorylation of MAP-kinase. It is plausible that PI3K contributes to the phosphorylation of ERK by contributing to the localization of PAK, thus facilitating PAK activation, and downstream ERK activation.

Other potential mechanisms for the role of PI3K in paxillin phosphorylation are implied by studies using paxillin knockout mice. Fibroblasts from the paxillin KOs have abnormal focal adhesions, decreased cell migration, inefficient localization and phosphorylation of FAK, and decreased cell spreading on fibronectin. The contribution of paxillin to focal adhesions comes as no surprise, however the finding that paxillin deficient fibroblasts are impaired in cell spreading is somewhat intriguing as it suggests that paxillin may be an effector of Rac, a G-protein directly implicated in T cell spreading (169). In this pathway paxillin phosphorylation may depend on PI3K as a result of PIP3-dependent activation of an unknown GEF, upstream of Rac. A simple way to address this possibility would be to examine the phosphorylation of paxillin in Rac deficient mice.

5.3.2 Future directions

Future directions in this area include studies addressing whether or not paxillin physically associates with p95PKL and Pix β. If the complex exists, addressing whether the phosphorylation of PKL or Pix β is dependent on PI3K activity would be our next step. Another pertinent study involves the direct assessment of whether or not the localization of paxillin, PKL or Pix β is PI3K dependent. These studies could be done using confocal microscopy, western blotting and immunoprecipitation techniques.

That paxillin is required for cytoskeletal rearrangement is well documented; however it remains to be clarified if phosphorylation is required for paxillin-mediated cell processes. A preliminary approach to answering this

question would be to design a dominant negative mutant of paxillin that is incapable of being phosphorylated, and test the effects of this mutant on focal adhesion formation and cell spreading. Another important question that needs to be answered is: what protein is phosphorylating paxillin. Simply putting purified PI3K together with paxillin *in vitro* may answer this question. Perhaps most important of this line of research is to address if paxillin phosphorylation is ablated or impaired in PI3K KO mice.

5.4 PI3K plays a role in CTL Spreading

Our data suggests that PI3K is activated upstream of cytoskeletal rearrangement and cell spreading; AKT is phosphorylated in CTL stimulated with immobilized anti-CD3 as well as CTL stimulated with cross-linked anti-CD3 (data not shown). Moreover, our results show that PI3K activity is required for CTL spreading on immobilized anti-CD3 antibody; CTL spread and become flattened after approximately 20 minutes, when stimulated with immobilized anti-CD3 antibody, however this rapid spreading does not occur when CTL are treated with PI3K inhibitors.

The mechanical process of cell spreading is correlated with sustained tyrosine phosphorylation; membrane proximal proteins in T cells stimulated with immobilized anti-CD3 undergo sustained tyrosine phosphorylation whereas proteins in T cells stimulated with cross-linked anti-CD3 do not. Moreover, sustained tyrosine phosphorylation facilitates effector function, whereas transient phosphorylation does not (61). Previous studies have

implicated PI3K in cell spreading in other cell types. Lowell *et al.* have shown that blocking PI3K activity inhibits macrophage spreading on fibronectin (145). As well, treatment of platelets with PI3K inhibitors results in inhibition of normal actin cytoskeletal rearrangements (146). Cell spreading could be one consequence of PI3K involvement in the localization and phosphorylation of proteins involved in cytoskeletal rearrangement.

5.4.1 Potential Mechanism for the involvement of PI3K in CTL spreading

Previous studies have shown associations between PIP3 in the membrane and actin polymerization; PI3K localizes to the membrane upon ligand stimulation (170), where it becomes important for interactions with the actin cytoskeleton (145). Haugh et al used a GFP-AKT fusion protein to show that PIP3 localizes to the contact site between fibroblasts and the ECM, defining a peripheral zone within which actin is preferentially polarized (171). This study suggests that upon CTL activation, the generation of PIP3 may directly facilitate actin polymerization. Alternatively, PIP3 is known to contribute to the localization and activation of WASp, a cytoskeleton associated protein known to interact with Apr2/3 and actin filaments. Thus PI3K activity could play a role in cell spreading through the localization and activation of WASp. WASp relocation to the membrane in a PIP3 dependent manner, adjacent to the cytoplasmic portion of a ligated signalling receptor protein tyrosine kinase, could induce actin polymerization and cell spreading.

The G-protein Rac has also been implicated in T cell spreading (169); active Rac mutants, transfected into Jurkats induce cell spreading on fibronectin in a wortmannin and Ly294002 sensitive manner. Interestingly the researchers argue that, because expressing a membrane associated form of PI3K in Jurkat fails to induce cell spreading, the sensitivity of Rac-induced spreading to PI3K inhibitors is a result of drug-specificity overlap to another PI kinase. This argument can be refuted by evidence suggesting that PI3K is constitutively active in Jurkat, thus transfecting in more PI3K should have little effect on the cell (172). A means to clarify this argument is to cotransfect Rac together with membrane associated PI3K into Rac KO T cells and look for augmented cell spreading.

5.4.2 Future Directions

As an extension to our studies addressing CTL spreading on immobilized anti-CD3 after 20 minutes, we also addressed if spreading is completely inhibited over a longer period of time, or if spreading is simply delayed when PI3K activity is inhibited. Our preliminary results suggest that in fact, spreading delayed, supporting a role for PI3K in optimizing the cytoskeletal events involved in cell spreading (data not shown). On an intracellular level, this suggests that PI3K activity, and the generation of PIP3, contribute to but are not the only mechanisms of localization and regulation of proteins that facilitate cell spreading.

Another area of study that we addressed is whether or not actin polymerization is inhibited in CTL treated with PI3K inhibitors and stimulated

with target cells. Although a role for PI3K in AB.1 actin polymerization is likely, actin polymerization only occurs in conjugates. Thus, where conjugate formation was inhibited, so was actin polymerization. This suggests that contact and conjugate formation precedes actin polymerization in our CTL, and that the role of PI3K in actin polymerization cannot be assessed using this system.

Other questions that should be addressed include assessing the role for PI3K in CTL spreading on fibronectin in concert with immobilized anti-CD3, as well as CTL spreading on immobilized anti-CD3 with immobilized ICAM. These studies would increase the validity of a role for PI3K in cell spreading as an extension of what is seen *in vivo*; stimulation and the induction of cell spreading as a result of integrin ligation followed by TCR ligation would more resemble what is seen *in vivo*. An even more poignant question is whether or not cell spreading is impaired in PI3K KO T cells.

5.5 PI3K plays a role in CTL Adhesion to target cells

Our results suggest that PI3K activity is required for CTL adhesion to target cells; wortmannin treatment inhibits the formation of AB.1 CTL/ $L1210^{KB/DD}$ conjugates. Adhesion of the T cell to a target cell is critical for initiating T cell activation and effector function. Adhesion results from LFA-1/ICAM interactions, as well as from CD8 interaction with MHC class I; these interactions facilitate T cell/ target cell tethering allowing the TCR to survey the target for antigen. If the right antigen is present, the T cell becomes

activated in response to TCR/coreceptor signalling. To further stabilize the interaction between an activated T cell and a target, TCR signalling triggers inside out signalling events that increase the affinity of LFA-1/ICAM interactions. This increased affinity and stability facilitates long lasting conjugation resulting in sustained signalling, CTL activation, and degranulation.

Previous studies supporting a role for PI3K in adhesion include a study done in 1997 by King *et al.*, which showed that plating fibroblasts on fibronectin-coated surfaces caused the clustering of surface integrins, leading to enhanced PI3K activation (152) suggesting that adhesion and PI3K activation are interrelated. More recently, studies suggest a role for PI3K in the inside-out signaling that facilitates tight conjugate formation (155), (151), (156). A consequence of PI3K involvement in the recruitment and phosphorylation/regulation of proteins may be that PI3K contributes to localization and activation of proteins involved in adhesion.

5.5.1 Mechanism of PI3K involvement in Adhesion

CTL conjugation is mediated by LFA-1 and CD8 to different extents. In T lymphocytes, signalling through a variety of receptors including the T cell receptor and CD28 results in the induction of integrin activation (147), (148), (149) and increases the affinity of CD8 for class I MHC (41). At the same time, signalling through these receptors results in the recruitment and activation of PI3K at the membrane. Thus, we, and others (150) have

postulated that PI3K participates in signalling events that mediate inside out signalling, resulting in integrin activation and CD8 affinity enhancement.

One possible mechanism of PI3K involvement in inside-out signalling may involve Vav-1. Vav-1 has been implicated as an important signalling component in many aspects of cytoskeletal rearrangement, including inside-out signalling; peripheral T cells in $Vav^{-/-}$ deficient mice are unable to form conjugates with peptide-loaded APCs (173). PI3K has been implicated in the recruitment and activation of Vav-1. Although we do not observe PI3K dependent phosphorylation of Vav-1 when our CTL are stimulated with immobilized anti-CD3 antibody, we do see inhibition of conjugate formation between CTL and specific targets. Perhaps some of the inside out signals that result from TCR together with LFA-1 signalling trigger Vav-1 phosphorylation in a PI3K dependent manner, and result in increased LFA-1 avidity for its ligand. By stimulating with immobilized anti-CD3 alone, we bypass the need for LFA-1 mediated adhesion altogether.

Recent studies further implicate PI3K in inside out signalling; cross-linking either α-4 integrins or the CD3 on Jurkat cells induces adhesion to ICAM-1 in a PI3K dependent manner (155). A potential mechanism for the involvement of PI3K in increased adhesion may involve cytohesin-1. Studies on cytohesin-1, a cytoplasmic regulator of β2 integrin adhesion to ICAM-1, have shown that cytohesin-1 recruitment to the cytoplasmic domain of LFA-1 is PI3K dependent (151). Cytohesin-1 contains a PH domain that specifically interacts with PIP3 in the membrane facilitating not only compartmentalization

for cytohesin-1, but also contributing to the specificity of the GEF activity of cytohesin-1 (151), (153), (154). These data suggest that PI3K is an upstream regulator of cytohesin-1 and as such may contribute to inside out signalling and cell/cell adhesion.

PI3K may also contribute to adhesion by signalling the avidity enhancement of CD8. Mescher *et al.* have found evidence supporting a role for PI3K in the signalling events that initiate with TCR stimulation and result in the up-regulation of CD8 dependent adhesion (156). This data implicates PI3K in the regulation of an unknown cytoplasmic regulator of CD8 adhesion.

Taken together, these results strongly suggest that PI3K is an important regulator of integrin-mediated adhesion as well as a participant in the inside-out signalling events that trigger avidity enhancement of CD8.

5.5.2 Future Directions

An important question that needs to be answered with regard to the involvement of PI3K in adhesion is the mechanism. Although it is likely that PI3K regulates the localization of cytoplasmic regulators of LFA-1 adhesion, such as cytohesin-1, the PIP3/cytohesin-1 interaction must be confirmed in our CTL clones. One key area of research includes addressing the expression, phosphorylation, and localization of cytohesin-1, under conditions of PI3K activity and inactivity. As well, it is likely that PI3K contributes to the localization and phosphorylation of other players involved in adhesion. Taking a closer look at some of the less-well defined proteins that contain PH domains, using dominant negative and knockout studies in T cells and

specifically addressing adhesion, could help to flesh out other proteins that play a role in inside-out signaling in a PI3K dependent manner.

5.6 PI3K plays a role in the Phosphorylation of PKC θ

Our results show that PI3K activity is required for the phosphorylation of PKC θ; PKC θ is phosphorylated upon CTL stimulation with immobilized anti-CD3 antibodies and with target cells, phosphorylation is inhibited when CTL are pretreated with PI3K inhibitors. As well, PKC θ phosphorylation is dependent on the integrity of the cytoskeleton; PKC θ becomes dephosphorylated in CTL stimulated with immobilized anti-CD3 and treated after 15 minutes with Cytochalsin E.

PKC θ is a novel PKC isoform, highly expressed in T cells, that cooperates with calcineurin to induce transcription of IL2 (100). PKC θ is potentially linked to cytoskeletal rearrangement in that it is functionally associated with Vav (95), and it is recruited to the center of the immune synapse in a Vav-dependent and actin polymerization-dependent manner (105). Significantly, PI3K has recently been implicated in the localization of PKCθ to the membrane in Jurkat cells; PI3K activity is required for membrane/cytoskeleton recruitment of PKC θ as recruitment is impaired when PI3K inhibitors are employed (174). PKC θ relocalization and phosphorylation in a PI3K dependent manner may result in a role for PKC θ as a molecular bridge between cytoskeletal rearrangements and downstream signaling events that induce CTL effector function.

5.6.1 Mechanism of Involvement of PI3K in PKC θ Phosphorylation and Localization

PKC θ has been shown to functionally associate and in some systems to physically associate with Vav-1 (95) (175). As well, Villalba and colleagues have shown that costimulation of Jurkat with anti-CD3 and anti-CD28 induces translocation of PKC θ from the cytosol to membrane (105). Given that the PH domain of Vav has been implicated in Vav localization and phosphorylation at the membrane (168), it makes sense to suggest that PIP3 interaction with Vav and correspondingly with PKC θ facilitates its localization to the membrane where phosphorylation can take place. In support of this mechanism, PKC θ recruitment to the membrane is impaired in Vav deficient cells (174). Additionally PKC θ has been found to associate with Fyn, which is interesting because Fyn was recently identified as the tyrosine kinase that plays a major role in phosphorylation and activation of Vav under physiological conditions of antigen stimulation (176). Consistent with this finding, Fyn-deficient T cells have decreased responses in terms of Vav and PKC θ activity (177). Given the implication of PIP3 in Vav localization and phosphorylation a Fyn/Vav/PKC θ functional interaction fits nicely into a PI3K dependent model for PKC θ phosphorylation. However, such an interaction remains to be seen; a physical association between Vav-1 and PKC θ in activated mature T cells has yet to be observed, moreover it does not seem to occur in AB.1 CTL clones, stimulated with immobilized anti-CD3. Thus it is unlikely that the dependence of PKC θ on PI3K activity has any direct relation

to Vav-1, unless PKC θ phosphorylation is dependent on Vav-1-dependent cytoskeletal rearrangement. Our results show that the phosphorylation of PKC is dependent on the integrity of the cytoskeleton. Others have shown that PKC localization to the center of the immune synapse is dependent on Vav-dependent actin polymerization (105). Thus the PKC θ/Vav connection may be indirect and PI3K may play a role in bridging Vav-1 and the cytoskeleton, thus enabling the phosphorylation and localization of PKC θ.

There are also potential mechanisms that would implicate PI3K in a more direct role in the phosphorylation and localization of PKC θ. For example, PKC θ has also been shown to physically interact with PDK-1 in fibroblasts (178). This type of interaction could facilitate the localization of PKC θ to the membrane in T cells as well as the phosphorylation of PKC θ at the membrane. In Jurkat, PDK-1 has been shown to indirectly affect the localization of PKC (174) although a direct association in this cell type does not occur. Whether or not a PDK-1/PKC θ interaction occurs in our CTL clones is unknown, thus a PKC/PDK-1 interaction remains a potential mechanism.

Recent studies have also shown that PKC θ and PKB/AKT can functionally and constitutively associate with one another in Jurkat cells, as well as directly bind to each other *in vitro* (179). Thus an AKT/PKC θ interaction could be the mechanism by which PKC θ is localized to the membrane and phosphorylated in a PI3K dependent manner; PI3K-activated AKT could recruit and phosphorylate PKC θ. However, in our AB.1 CTL

clones, we cannot detect a PKC θ/AKT association under immobilized anti-CD3 stimulatory conditions; meanwhile the phosphorylation of PKC θ is still PI3K dependent. Thus an AKT/PKC interaction is not likely to be the mechanism of PKC phosphorylation in our cells.

In contrast to what is seen in Jurkat, PKC θ in our CTL seems to be constitutively at the membrane under both basal and stimulated conditions; it is only the phosphorylation of PKC that increases with stimulation. However, PKC θ, already at the membrane, may be phosphorylated by a kinase whose localization or activation is dependent on PI3K and the generation of PIP3. Again a potential candidate is PDK-1. PDK-1 has been shown to phosphorylate other PKC isoforms (180). PI3K-dependent phosphorylation of PKC θ may result in the relocation of PKC θ to the center of the immune synapse, but this remains to be seen.

Taken together, these results suggest that PI3K plays a role in the phosphorylation of PKC θ and thus, may participate in bridging cytoskeletal rearrangement and the downstream signalling events that facilitate CTL degranulation. The nature of this role may be indirect, through contribution to cytoskeletal rearrangement for which sustained PKC θ phosphorylation is dependent, or direct through the generation of PIP3, which may facilitate the recruitment and activation of kinases that associate with and or phosphorylate PKC θ.

5.6.2 Future Directions

Some important questions that need to be addressed with regard to the role of PI3K in PKC θ phosphorylation include: 1) Do PKC θ and PDK-1 physically associate? 2) Does the phosphorylation of PKC θ imply kinase activity, an alternative activity, localization and/or regulation? 3) What lies directly upstream of PKC θ with regard to the phosphorylation of PKC θ? 4) If PI3K plays a role in PKC θ localization does it contribute to the localization of PKC to the centre of the immune synapse, or does it direct PKC θ more generally to lipid rafts? And 5) is PKC θ phosphorylated and does it localize to the membrane in PI3K KO T cells.

5.7 Summary and Model for PI3K involvement in CTL effector function

PI3K activity is required for CTL activation and plays a role in the cytoskeletal events that are required for effector function. We suggest a model (outlined in figure 25) whereby PI3K is activated in response to TCR signaling and correspondingly contributes to the sustained signaling of cytoskeletal associated proteins including paxillin, and proteins involved in cell spreading, as well as adhesion. As well PI3K-dependent cytoskeletal rearrangement facilitates the phosphorylation of PKC θ, and may contribute to the localization of PKC θ to lipid rafts, or to the center of the immune synapse. The result of PI3K activity in this sequence of molecular events is contribution to CTL degranulation; the data outlined in this thesis provides

new insight into the critical nature of PI3K and its lipid byproduct PIP3 in CTL signaling and cytoskeletal rearrangement, facilitating CTL degranulation.

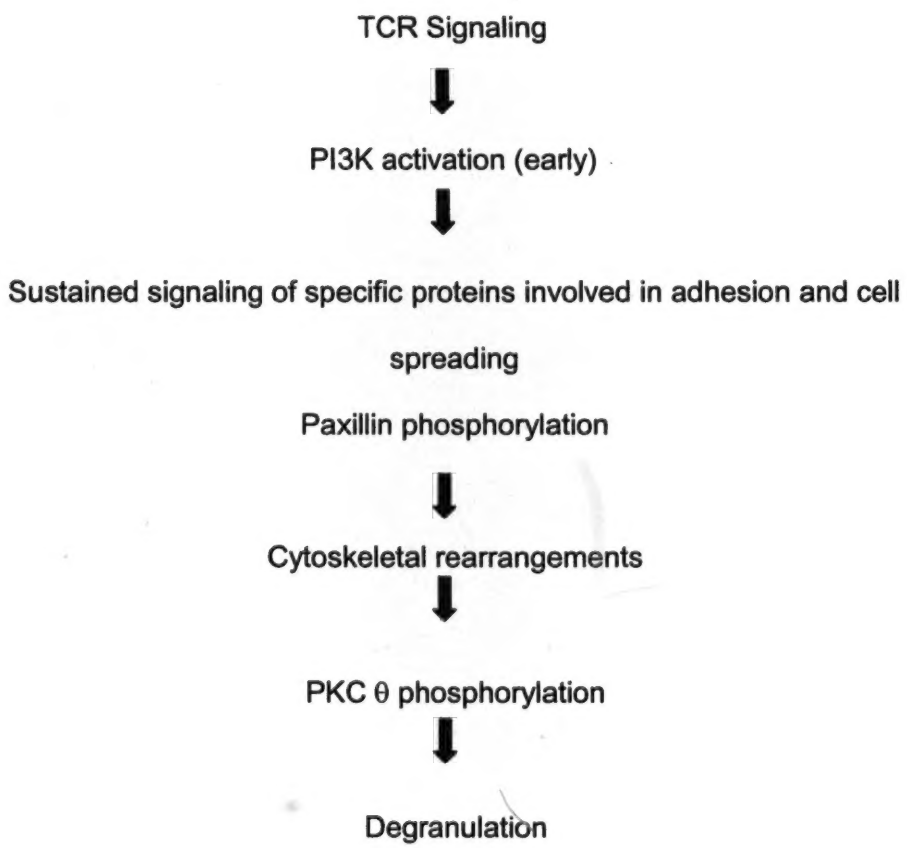


Figure 24. **Model: PI3K in CTL activation**

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Appendix

A.1 Wortmannin has no adverse effect on the viability of CTL

Another critical control with regard to using drug inhibitors to inhibit PI3K was to consider the effect of these drugs on cell viability. Potent effects on cell survival would outweigh any other observations that would result from drug treatment such as cytoskeletal rearrangements or the lack there of, of alterations in signaling events etc. Thus we performed an experiment to determine the effect of drug treatment at various concentrations on cell viability. CTL were treated for 30 minutes, or not, and then stimulated with immobilized anti-CD3 antibodies, of left unstimulated. The results in Appendix figure 1 suggest that wortmannin has no effect on cell viability over a period of the two days (24 hours).

A.2 LY 294002 inhibits degranulation of CTL as stimulated by target cells

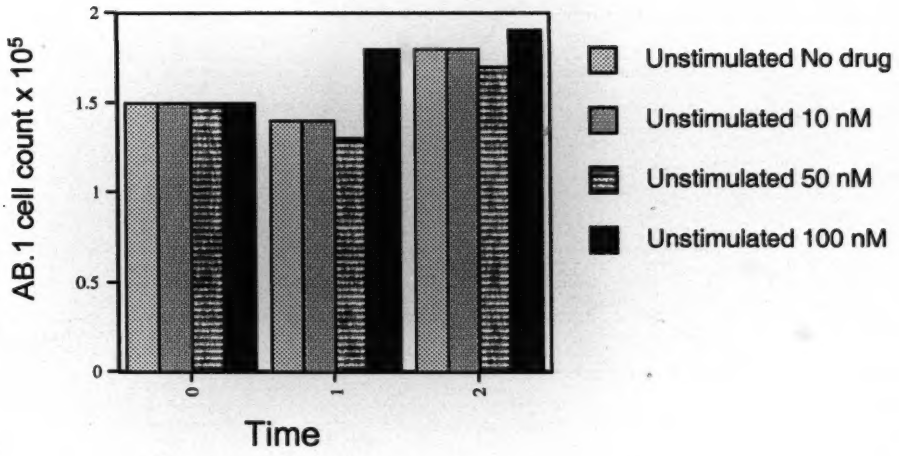
CTL, stimulated with antigen bearing target cells (L1210 $^{Kb/Dd}$), were also inhibited with Ly 294002. As in the case of wortmannin inhibition of PI3K, CTL degranulation was inhibited to the same extent as when CTL were stimulated with anti-CD3 (Appendix figure 2). The data in figure 2 represent one of three identical experiments where AB.1 were stimulated with allo targets and Ly 294002 was used to inhibit PI3K activity. These results

suggest that PI3K is a critical player in the intracellular events that lead to CTL effector function.

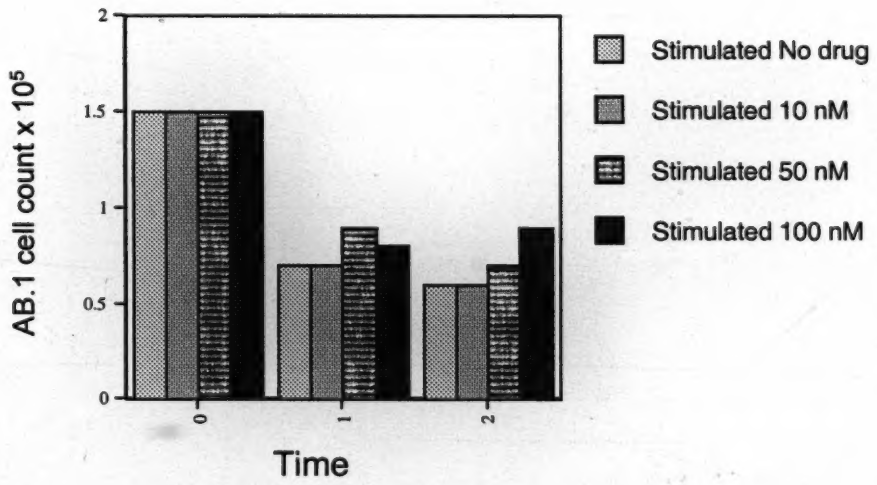
A.3 PKC θ is constitutively at the membrane in unstimulated AB.1 CTL clones

CTL were stimulated by coculture with target cells for 5 minutes. Cell lysates were fractioned into crude, soluble, membrane and cytoskeletal components via ultra-centrifugation methods and Triton X 100 solublization. These fractions were then analyzed through western blotting to assess the localization of PKC θ. Appendix figure 3 shows the localization of PKC θ in the membrane fraction.

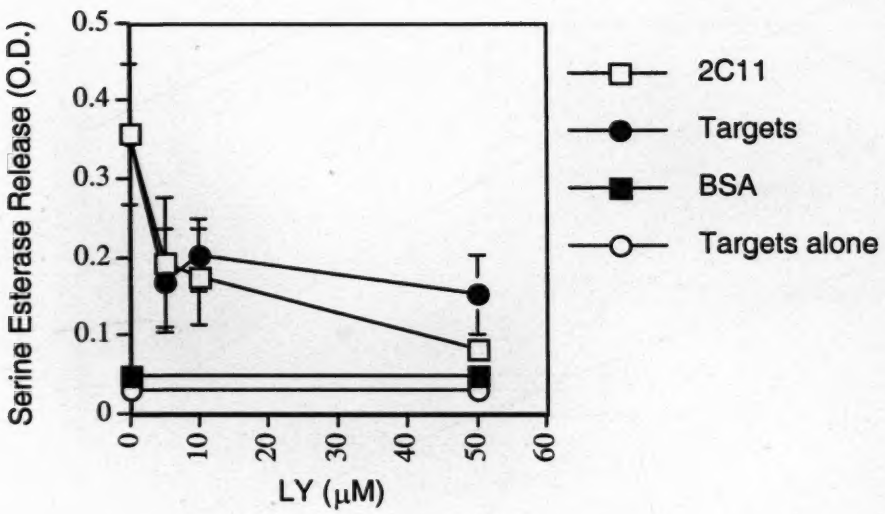
A.



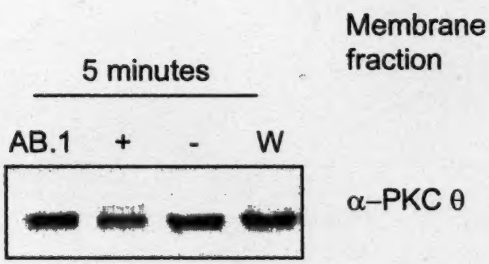
B.



Appendix Figure 1. **Wortmannin treatment has no effect on AB.1 viability under basal conditions or upon stimulation.** AB.1 CTL clones were treated with indicated concentrations of wortmannin for 30 minutes at 37 °C and stimulated with immobilized 145-2C11 antibodies (figure B) or left unstimulated (Figure A). Live cells were counted after 4 hours (time 1) and 24 hours (time 2).



Appendix Figure 2. . **Ly 294002 Treatment inhibits Degranulation regardless of Stimulation through CD3 or with Target cells.** AB.1 CTL clones were pretreated with Ly 294002 for 30 minutes at 37°C at the indicated concentrations and stimulated with either 145-2C11 (anti-CD3) or with target cells bearing the relevant MHC (L1210 K^b/D^d). Stimulation lasted 4.5 hours, after which the supernatants were collected and assayed for serine esterase as an indicator of degranulation. ((-□-) AB.1, pretreated with LY294002 and stimulated with 145-2C1, (-●-) AB.1 pretreated with LY294002 and stimulated with targets, (-■-) AB.1 left unstimulated,(-O-) target cells alone.)



Appendix Figure 3. **PKC θ is present in the membrane in unstimulated AB.1.** AB.1 CTL clones were stimulated by coculture with target cells for the indicated time period. Cells were lysed and post nuclear cell lysates, separated into fractions. Reduced proteins in each fraction were blotted with antibody for PKC θ. ((AB.1) AB.1 alone without stimulation, (+) AB.1 + L1210 $^{kb/Dd}$, (-) AB.1 + L1210, (W) AB.1 + L1210 $^{kb/Dd}$ + 50 nM wortmannin.)